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SOURCES OF RESISTANCE IN ALFALFA TO THE NORTHERN ROOT-KNOT NEMATODE, MELOIDOGYNE HAPLA [1]

E. H. Stanford, B. P. Goplen, and M. W. Allen

SUMMARY

Of 21 varieties of alfalfa, 5 related *Medicago* species, 54 Foreign Plant Introductions, and 200 lines of material from the California breeding program, only the alfalfa variety Vernal and the local Common strain Hilmar contained plants highly resistant to a virulent collection of *Meloidogyne hapla*. Progeny tests showed transmission of resistance to the offspring. These selections coming from cultivated varieties are suitable materials for use in a breeding program.

The northern root-knot nematode, *Meloidogyne hapla* Chitwood, frequently attacks alfalfa in the lighter soils of the Central Valley of California. An accurate evaluation of the damage is difficult. In some very heavily infested fields, seedling growth is slow, and, consequently, practically no hay is produced during the first season. This is not surprising, in view of the restricted root development and almost complete absence of a tap-root system, which have been observed by the authors and have been reported by Wilhite and Smith[2] and Thorne.[3] If the fields are well cared for during the first season, satisfactory stands can usually be obtained, with nearly normal production in the second season. During the cool, winter months, when the nematodes are less active, the plants are apparently able to develop more extensive root systems, capable of supporting the plant. The effect of this nematode has not been established, but there is some indication that it may be a factor in shortening the life of an alfalfa stand. The prevalence of this nematode in the interior valley of California, where alfalfa is one of the leading crops, suggests that resistance might profitably be incorporated into the commercially grown alfalfa varieties. Preliminary to launching such a breeding program, a survey was made to find sources of resistance. Reynolds[4] reported on resistance in alfalfa to *M. javanica* (Treub) Chitwood and *M. incognita acrita* Chitwood, but no reports on resistance to *M. hapla* have been noted.

MATERIALS AND METHODS.—A preliminary test showed that a uniform, high degree of galling could be secured in the greenhouse. Since field tests with the nematode are notably erratic, all tests were carried out in greenhouse benches. A collection of the nematode identified as *M. hapla*, collected near Caruthers, California, was used as a source of inoculum in all tests. This collection had previously been found to produce heavy galling on alfalfa.

In preparation for the testing, a greenhouse bench was filled 6 in. deep with a 1:1 steam-sterilized mixture of Yolo sandy loam and fine sand. A soil-heating cable with a thermostatic control was placed 1 in. from the bottom of the bench and set to maintain a soil temperature of 28°C. The nematode inoculum was built up on the roots of tomatoes. The heavily galled roots were chopped up, placed in furrows spaced 3 in. apart across the bench, and covered with about ½ in. of soil. The alfalfa seed was then planted and covered with an additional ½ in. of soil. About 50 seeds of each variety were planted in 2-ft. rows in the bench. In the first test, every third row was planted to the variety California Common to serve as a check on level of infection. Galling was so heavy

TABLE 1.—*Susceptibility and resistance of varieties and species of alfalfa to Meloidogyne hapla*

Variety	Resistance rating [a]
California Common	S
Caliverde	S
African	S
Grimm	S
Hilmar	7R. 155S.
Williamsburg	S
Hairy Peruvian	S
Lahontan	S
California Common 49	S
Atlantic	S
Ladak	S
Buffalo	S
Narragansett	S
Rhizoma	S
Ranger	S
India	S
Du Puits	S
Arizona A-4-35	S
Talent	S
Nomad	S
Vernal	26R. 87S.
M. lupulina	S
M. glutinosa	S
M. falcata	S
M. gaetula	S
M. marina	S

[a] S, susceptible; R, resistant. Numbers indicate number of plants of each classification. If no numbers are given, all plants in the row were of the rating indicated.

[1] Accepted for publication January 28, 1958.

[2] Wilhite, F. M., and O. F. Smith. 1949. Root-knot nematode control as a factor in the study of fertility levels on the production of alfalfa hay. Nevada Univ. Agr. Expt. Sta. Research Rept. No. 156.

[3] Thorne, G. 1948. Nematodes as a disturbance factor in greenhouse, plot and field experiments. U. S. Dept. Agr. Plant Disease Reptr. 32: 473-475.

[4] Reynolds, H. W. 1955. Varietal susceptibility of alfalfa to two species of root-knot nematodes. Phytopathology 45: 70-72.

and uniform on all the check rows that checks in subsequent tests were planted in every tenth row. After the first and following tests, the galled roots of the alfalfa plants were chopped up and returned to the bench to serve as inoculum for the succeeding test. Tops of the plants were trimmed back about 2 months after planting, and the plants were dug and classified for degree of infection 3 months after planting. Since the galls are easily broken off, the roots were dug with considerable dirt adhering, and carefully washed in a large can of water.

Plants were rated on a scale of 0–4, according to the number and size of galls, ranging from no evidence of infection to heavy infection. Some weak plants, with poorly developed root systems, were difficult to classify, but strong, vigorous plants were easily classified.

Results.—In all tests, the check rows were 100 per cent infected, with infection comparable to that of the Caliverde plants in Fig. 1.A.

Table 1 gives nematode-resistance ratings of 21 alfalfa varieties and 5 related species of *Medicago*. Ratings of 0-1 were considered resistant; 2–4 ratings were considered susceptible. Table 2 lists the Plant Introductions tested, all of which were susceptible. In addition, 200 lines of breeding material in the California program were tested, and all were completely susceptible. It will be seen from Table 1 that only the variety Vernal and the local strain Hilmar gave any resistant plants. Vernal gave 23 per cent resistant plants; Hilmar gave 4.3 per cent. Some gradation occurred in the degree of susceptibility in this material, but those rated as resistant were almost entirely free of galls. Tests of selfed progenies showed that this resistance is genetic, since resistant plants consistently produced some resistant plants in their offspring. Fig. 1.B shows a representative selfed progeny of the selection M-4 from the variety Vernal. Resistant plants in this line were completely free of galls, and only 1 plant was susceptible of 403 tested.

Discussion.—While resistance to *M. hapla* seems relatively rare in alfalfa, individual plants of the variety Vernal and the strain Hilmar are highly resistant. This resistance is transmitted to the offspring, and could be readily used in a breeding program. An alfalfa variety with resistance may not only give higher

Fig. 1. Alfalfa roots showing resistance and susceptibility to northern root-knot nematode. A) Caliverde variety, with heavy galling and restricted tap-root development. B) Progeny of selection M-4 from the variety Vernal, with normal root development.

TABLE 2.—*List of alfalfa plant introductions found susceptible to M. hapla*

P.I. No.	Origin	P.I. No.	Origin
163107	India	202733	Portugal
164415	India	202734	Portugal
177012	Turkey	202735	Portugal
177013	Turkey	202736	Portugal
177464	Turkey	202738	Portugal
177980	Turkey	202739	Portugal
178981	Turkey	207495	Afghanistan
179946	India	211054	Afghanistan
180303	India	211606	Afghanistan
181915	Syria	211607	Afghanistan
181889	Syria	211608	Afghanistan
182242	Turkey	211609	Afghanistan
183695	Turkey	211610	Afghanistan
183261	Arabia	212104	Afghanistan
197829	Algeria	212105	Afghanistan
199273	Poland	212106	Afghanistan
199275	Poland	212612	Afghanistan
199276	Poland	217419	Denmark
199277	Poland	217648	Iraq
199278	Poland	219927	Afghanistan
199279	Poland	219928	Afghanistan
199280	Poland	220298	Afghanistan
199281	Poland	220299	Afghanistan
202729	Portugal	220300	Afghanistan
202730	Portugal	220301	Afghanistan
202731	Portugal	220530	Afghanistan
202732	Portugal	220668	Afghanistan

hay yields but may also benefit following crops that are susceptible, such as tomatoes. While these tests showed the varieties African and Chilean to be susceptible to *M. hapla*, Reynolds[4] found these varieties were resistant to *M. javanica* and *M. incognita acrita*. Hare,[5] working with peppers, similarly found that varieties resistant to *M. javanica* and *M. incognita acrita* were susceptible to *M. hapla*. This indicates that the basis for resistance differs with the different species of nematodes. The resistant stocks reported here have not yet been tested against either of those species, but it is probable that breeding for resistance to the various nematode species will need to be carried on as separate projects. Work is now under way to test our resistant material against a number of collections of nematodes to determine if the resistance is maintained against these collections. Genetic studies have been initiated to determine the mode of inheritance of resistance.

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RESPIRATORY RESPONSES AND SYSTEMIC EFFECTS IN FUSARIUM-INFECTED TOMATO PLANTS[1]

R. P. Collins and R. P. Scheffer

SUMMARY

Respiration in leaves of tomato plants was stimulated soon after infection with *Fusarium oxysporum* f. *lycopersici*. Respiration in diseased leaves reached a peak 9–14 days after inoculation, and was sometimes double that of healthy controls. Stems responded similarly. Results were comparable whether calculated on a dry-weight or nitrogen-content basis, even though total nitrogen at later stages was higher in diseased tissue than in healthy controls. Since leaf blades were not invaded directly by the pathogen, these results suggest systemic toxemia. Respiratory toxins are postulated; these should not be confused with wilt-inducing agents. Permeability changes in pathogen-free leaves of infected plants further support the systemic-toxemia hypothesis. Ethylene, fusarinic acid, and probably pectic enzymes were eliminated as the substances stimulating respiration, although these materials may have other roles in Fusarium infection. Ethylene hastened disease development when introduced in solution into inoculated susceptible cuttings, and caused cuttings of similarly treated resistant plants to become diseased.

Past work indicates that Fusarium wilt of tomato, caused by *Fusarium oxysporum* f. *lycopersici* (Sacc.) Snyder and Hansen, is a useful disease for the study of pathogenesis. The physiological reactions leading to wilt development (11, 18), vascular browning (6, 11), and epinasty (8) have been examined, but other pathological alterations have had little attention. Only Bloom (4) has considered the respiratory responses of diseased plants. He measured the respiration of whole leaves and cuttings by gas trains and gravimetric analysis of carbon dioxide. After comparisons of inoculated and noninoculated plants, he concluded that respiration was stimulated prior to symptom development. The present work began as a re-examination of Bloom's work with more exact methods, followed by an extension of his observations. Possible changes in the permeability of leaf tissue were examined in parallel experiments. Several metabolites known to be involved in disease development were

[1] Accepted for publication February 3, 1958.
Michigan Agricultural Experiment Station Journal Article 2159. Photographic work by P. G. Coleman.

[5] Hare, W. W. 1957. Inheritance of resistance to root-knot nematodes in pepper. Phytopathology 47: 455-459.

examined as possible causes of respiratory alterations following infection, or "pathological respiration." Ethylene received special attention.

The respiration of plants infected with rusts and mildews has been studied carefully. In these cases an increase in respiration following infection is clearly evident, and diffusible toxins accounting for the increases have been postulated (1). Recently, intensive work has been done on the nature of the respiratory alterations in rust-infected plants (5, 12), in powdery-mildew-infected plants (15), and in sweet potato tissue infected with *Ceratostomella fimbriata* (Ell. & Halst.) J. A. Elliott (2, 3). Such work is often hindered by the difficulty of obtaining diseased tissue that is free of the pathogen. Pathogen-free tissue is easy to obtain from Fusarium-infected tomato plants, which should make them useful for further study of metabolic derangements.

MATERIALS AND METHODS.—The tomato variety Bonny Best was used in all experiments requiring Fusarium-susceptible plants; the variety Jefferson was used when Fusarium-resistant plants were needed. Plants were grown in sand in the greenhouse and watered with a balanced nutrient solution (9) or with a suitable concentration of a soluble commercial fertilizer. Plants 6–8 weeks old were used when cuttings were needed. Stems were cut under water with a razor blade and the cuttings placed in the treating solutions. Cuttings 8–10 in. long were used.

Fusarium oxysporum f. *lycopersici*, strain R5-6, was grown in 500-ml Erlenmeyer flasks in shake culture at about 25°C, using the medium of Dimond and Waggoner (8): casamino acids 1.5 g; yeast extract 1.0 g; KH_2PO_4 1.5 g; $MgSO_4 \cdot 7H_2O$ 1.0 g; glucose 15.0 g; A–Z solution (9) 0.2 ml; distilled water to 1000 ml (pH 5.5–5.6). Under these conditions fungus growth was largely bud cells.

To inoculate, plants were removed from the sand, and the roots were washed in tap water and dipped directly into a Fusarium bud-cell suspension. Plants were then reset in sand and kept under greenhouse conditions that favored disease development. Non-inoculated controls, in the same manner, were uprooted, dipped in water, and reset.

Inoculum for cuttings was prepared from shake cultures by filtration through cheesecloth, centrifugation, and filtration by suction through coarse filter paper to remove aggregates and mycelial fragments. Bud cell suspensions were adjusted to 50,000 spores per ml, and cuttings were allowed to take up the suspensions through the cut ends by transpirational pull. Cuttings were then rooted in sand. The method is essentially that of Scheffer and Walker (19).

Wilt was rated on an arbitrary basis by the method of Gallegly and Walker (9), from 0 = no wilt to 4 = severe wilt. Vascular browning was rated after Scheffer and Walker (19), from 0 = no browning to 4 = browning throughout the stem.

The Warburg apparatus was used to determine oxygen uptake, following in general the method of Klinker (13). Tomato leaves were carefully selected for uniformity, using the third or fourth leaf above the cotyledon. Disks cut from the leaves with a 6-mm cork borer were placed on moistened filter paper in the bottom of Warburg flasks (20 per 15-ml flask). Flasks were equilibrated for 30 minutes and oxygen uptake measured at 30°C in the dark. After two hours, when measurements of oxygen uptake were completed, leaf disks were removed from the flasks, dried 48 hours at 100°C, and weighed. Respiration was calculated as μl of oxygen uptake per mg dry weight of tissue or as oxygen uptake per mg nitrogen. In other experiments, leaf disks were suspended in M/30 potassium phosphate buffer solutions at pH 5.9 and vacuum infiltrated. Respiratory quotients were found by the standard method (21), in which carbon dioxide was absorbed in one of 2 paired flasks, and not in the other.

Stem respiration was measured by using slices about 0.75 mm thick, cut with a straight razor and a hand microtome from the second internode above the cotyledon. Slices were washed 20 minutes in M/30 potassium phosphate buffer at pH 5.9. Stem slices were then removed from the buffer solution, blotted on filter paper, and rapidly transferred to a Warburg flask that contained a disk of filter paper moistened with 0.1 ml of distilled water (20 slices per flask). All values reported are the averages for 3 flasks. This is essentially Klinker's method (13); it was used after preliminary experiments indicated that it was satisfactory for stem slices.

Total nitrogen was determined by the micro-Kjeldahl techniques of Ma and Zuazaga (14) and Pepkowitz and Shive (17). Finely ground plant material was digested with concentrated sulphuric acid, using selenium and a mixture of potassium sulphate and copper sulphate as a catalyst. Ammonia was distilled into a boric acid solution containing a mixed indicator. This was titrated to a pink color, using 0.02 N hydrochloric acid. Each determination involved 24 flasks, 12 containing material from healthy plants and 12 containing material from diseased plants.

Possible changes in cell permeability were measured by conductivity of leaching fluids from healthy and diseased plants. Leaves from healthy and diseased plants were carefully selected for uniformity in size and appearance, washed in double-distilled water, and placed in flasks containing 50 ml of double-glass-distilled water. To ensure that all leaves were wetted, they were either vacuum infiltrated or the flasks containing the leaves in water were placed on a reciprocal shaker. After 7 hours the leaves were removed, and conductivity readings on the water were made with a conductivity bridge (Model RC-16B1, Industrial Instruments, Inc.) and a conductivity cell having a cell constant of 0.1. All measurements were made at 25°C. Leaves were dried 48 hours at 100°C, and conductivity was calculated in micromhos per mg dry weight.

Saturated ethylene solutions were prepared by bubbling ethylene (pure grade, Phillips Petroleum Co.) through distilled water for 30 minutes at 18°C.

At this temperature 100 ml of water will hold about 0.015 ml of ethylene (20). As needed for the various experiments, the saturated solutions were diluted with distilled water. Ethylene-air mixtures were prepared by partially evacuating a desiccator jar of known volume and injecting a given volume of ethylene with a hypodermic syringe filled from the ethylene tank with the aid of a rubber serum cap. Injections into the desiccator were made through a serum cap placed over the opening in the side arm. After the ethylene was added, the desiccator was brought back to atmospheric pressure and resealed.

RESULTS.—*Respiratory response of leaves to infection.*—The respiration of leaf disks was compared for healthy and diseased plants by 1 of 2 methods: 1) respiration of each of 4 inoculated plants was compared with respiration of each of 4 non-inoculated plants; 2) 1 leaflet was collected from each of 16–30 inoculated plants, and 5 disks were cut from each leaf. The disks were mixed together, and the respiration was compared with that of a comparable composite sample from non-inoculated plants, using 2–4 flasks each, with 20 disks per flask. The particular basis used will be indicated below. In all cases, results were expressed as a ratio obtained by dividing the respiration value for diseased tissue by the value for otherwise comparable healthy tissue.

Plants inoculated 4 weeks after germination had an increase in respiration 7 days later, as compared with healthy control plants. Respiration of diseased leaf tissue increased sharply on the eleventh day, reached a peak 13 days after inoculation, and dropped off rapidly thereafter (Fig. 1,A). In a second experiment, 7-week-old plants were used. Respiration of inoculated plants was significantly higher than the respiration of healthy plants 2 days after inoculation. Leaves from infected plants had slightly higher respiration for 8 days, but on the tenth day a sharp increase occurred. The respiration rate continued to climb until the fourteenth day, when the experiment was ended (Fig. 1,A). Two days after inoculation there were no differences in appearance between diseased and healthy plants. Plants 8 days after inoculation showed epinasty, slight yellowing of the lower leaves, and stunting. Plants 14 days after inoculation were yellowed and stunted, and had some wilting of lower leaves. Fusarium was not isolated from the leaf blades of diseased or healthy plants, although repeated at-

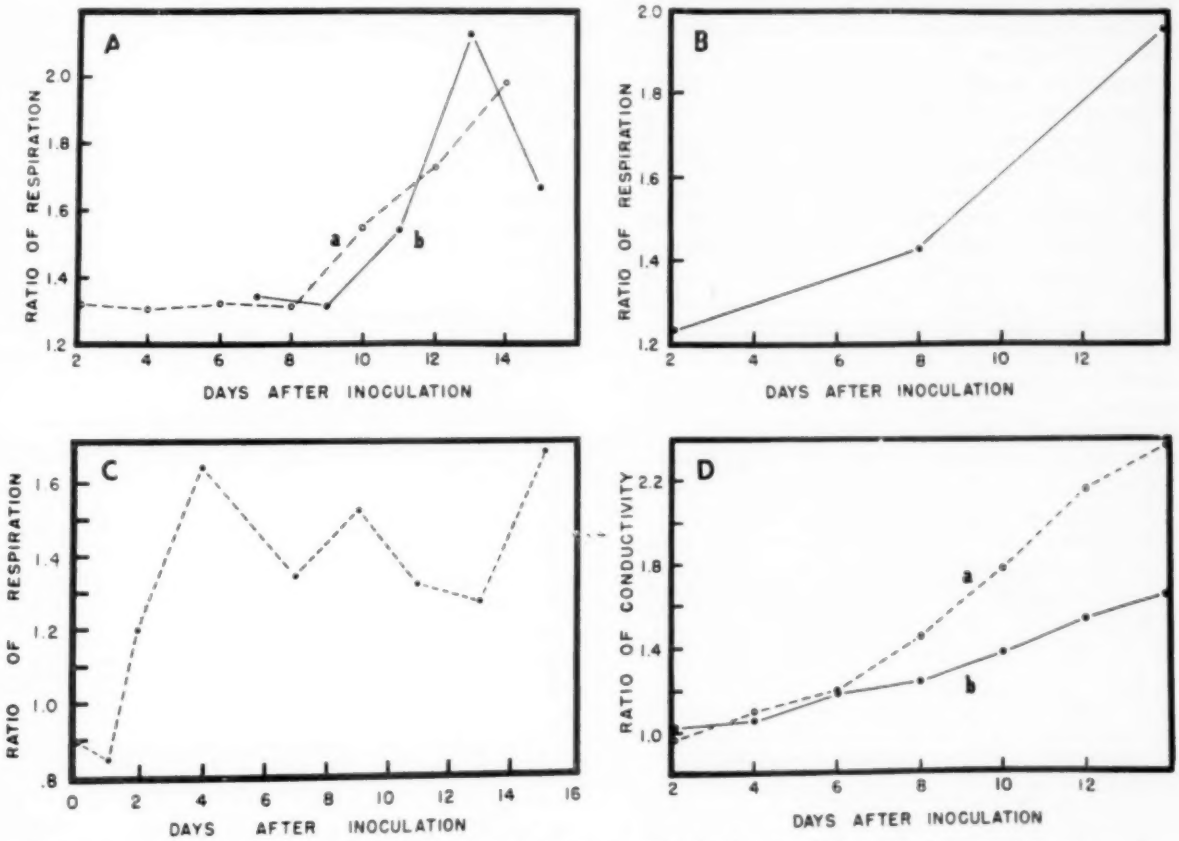

FIG. 1. Respiration and permeability changes in tissues as a result of infection. Values are the ratios between respiration or permeability in diseased plants and respiration or permeability in healthy plants. A) Respiration of leaf blades from 4-week-old (b) and 7-week-old (a) diseased plants, determined on a dry-weight basis. B) Respiratory changes in leaves of infected plants, determined on a total-nitrogen basis. C) Respiration of stem slices from diseased plants determined on a dry-weight basis. (D) Relative conductivity of leachates from diseased lower (a) and upper (b) leaves, determined on a dry-weight basis.

tempts were made, which indicated that the higher respiration was not due to respiration of the pathogen.

In another experiment a significant stimulation of respiration was noted in diseased plants as early as 1 day after inoculation, with 30, 41, and 20 per cent increases after 1, 2, and 3 days, respectively. The experiment was repeated, with essentially the same results, but in a later experiment the early stimulation failed to occur, suggesting that the reaction may be sensitive to environmental conditions.

In 2 further experiments, the respiration of leaf disks from diseased and healthy plants was determined as oxygen uptake per mg nitrogen. Leaves from healthy and diseased plants were collected 2, 8, and 14 days after inoculation. Oxygen uptake per unit of total nitrogen again showed that respiration in diseased plants was stimulated slightly after inoculation, and rose to almost double the value in healthy plants by 14 days after inoculation (Fig. 1,B). There were no differences in total nitrogen between diseased and healthy plants collected 2 and 8 days after inoculation, but leaves from diseased plants at 14 days had 10 per cent more total nitrogen. When the experiment was repeated, an 18 per cent increase in total nitrogen was found 15 days after inoculation. These differences were not enough to account for the increased respiration of diseased over healthy leaves.

Respiratory quotients (carbon dioxide production/oxygen uptake) were determined in 2 experiments at 2, 4, 6, 10, 17, and 19 days after inoculation. Quotients for leaves from diseased and healthy plants did not differ, and ranged from 0.91 to 0.97 (Table 1). Such values do not indicate differences in substrates or qualitative differences in respiration between diseased and healthy plants.

Results of 3 experiments with composite samples were comparable to those just described, except that with the composite sample the peaks in the respiratory curves for diseased plants were generally lower, with increase over the controls of 59–91 per cent. Respiration of diseased plants rose to a peak, then dropped, as compared to healthy controls. In one experiment the values were 0, 20, 76, 43, and 19 per cent increases 6, 8, 9, 13, and 15 days after inoculation. There was variation in oxygen uptake from one experiment to another; diseased tissue used 25.5–52.0 μl of oxygen per 100 mg dry weight per hour, while healthy tissue used 20.5–34.5. Presumably these differences are due to age of the leaves and environmental conditions of growth. These aspects are being studied further.

TABLE 1.—*Respiratory quotients (CO_2 produced/O_2 consumed) of diseased and healthy leaf tissue*

Days after inoculation	Respiratory quotients[a]	
	Healthy	Diseased
2	0.92	0.95
4	.95	.96
6	.97	.94
10	.95	.93
17	.94	.94
19	.91	.97

[a] Average value of 4 flasks each.

Since symptoms of Fusarium wilt usually start at the lower leaves and move up the plant, it seemed desirable to determine whether or not respiratory responses follow a similar pattern. The lower leaves of infected plants (node 3 above cotyledons) had 52 per cent higher respiration 11 days after inoculation than did the comparable healthy controls. Higher leaves on the same plants (node 7 above cotyledons) had a respiratory increase of only 22 per cent as a result of infection. These limited data suggest that respiratory responses move gradually up the plant as the severity of symptom expression increases.

Respiratory response of infected stems.—Oxygen uptake by stem slices was determined before inoculation and 1, 2, 4, 7, 9, 11, 13, and 15 days after inoculation. Respiration of infected plants increased on the second day after inoculation and remained at a higher level than that of healthy controls throughout the rest of the experiment (Fig. 1,C). The pattern was similar to that in leaves, except that high responses came earlier. These results were obtained during the winter, when light was low and root formation was poor, which may partly explain the erratic results.

Stem slices taken from the same regions as those used for respiratory measurements were placed on potato dextrose agar and incubated 1 week at 25°C. A few isolations of the pathogen were made 2 days after inoculation, but most sections did not contain the fungus until the seventh day. Increased respiration in stems after infection apparently results from a host response, and is not an additive effect of fungus respiration.

Respiratory response of leaves to fusarinic acid.—Possible effects of fusarinic acid are significant because it is a product of *F. oxysporum* f. *lycopersici* (16). A sample with a melting point of 106–107°C. obtained from Dr. F. H. Stodola, of the Northern Regional Laboratory (U.S.D.A.), was tested for its effect on tomato leaf respiration. Leaf disks were vacuum infiltrated with solutions of fusarinic acid, and oxygen uptake was determined manometrically. Four separate experiments were run, using concentrations from 10^{-2} to 10^{-5} M in potassium phosphate buffers (Table 2). Inhibition of respiration occurred with concentrations greater than 10^{-4} M, while solutions more dilute than this had no effect on respiration (Table 2). Fusarinic acid apparently is not involved in the respiratory reactions of infected plants.

Respiration of leaves from pectinase-treated cuttings.—Since pectic enzymes produced by Fusarium presumably have an important part in disease development (11), the effect of pectolytic enzymes on leaf respiration was determined. Commercial pectinase, a mixture of pectolytic enzymes (Nutritional Biochemicals), was used to avoid the complicating effects of other products produced by Fusarium. Tomato cuttings were allowed to take up solutions of pectinase for 3 days, following the procedure of Gothoskar et al (11). Five cuttings were treated with each concentra-

TABLE 2.—*Effect of various concentrations of fusarinic acid on respiration of tomato leaf disks*

Molar concentration	Respiratory ratio[a] at pH 6.0[b]	pH 5.0[b]
10^{-2}	45[c]	
10^{-3}	88	71
		97
5×10^{-4}		85
10^{-4}	96	
	87	
5×10^{-5}		98
10^{-5}	88	94
	99	
5×10^{-6}	103	
10^{-6}	99	97
		103
5×10^{-7}	92	
10^{-7}	90	101
		105
5×10^{-8}	111	
10^{-8}	96	108
		100

[a] Expressed as per cent of controls.
[b] At pH 6.0, M/30 KH_2PO_4–K_2HPO_4 buffer was used; at pH 5.0, M/30 KH_2PO_4 was used. In some cases, the latter varied somewhat from this pH value at the end of the experiment.
[c] Each value is the average for 2 flasks.

tion of pectinase, using 0.5, 0.25, and 0.125 per cent solutions and the heated control for each. Leaf disks were then cut and respiration determined manometrically. Pectinase treatment of cuttings did not affect leaf respiration in any case.

Respiration of ethylene-treated leaves and stems.—The effect of ethylene on the respiration of healthy tomato plants was tested in several ways. Ethylene solutions were prepared by saturating water with ethylene at 18°C and diluting this solution 1:10, 1:100, 1:500, and 1:1000 with distilled water. In one series of experiments, 8–10-in. cuttings were allowed to take up ethylene solutions for 3 days; the solutions were changed and the stem bases trimmed each day. Five cuttings were treated with each concentration, and cuttings were kept under artificial light at about 24°C. Controls were treated in the same way except that the stem bases were kept in distilled water. After 3 days, leaf disks and stem slices were cut and oxygen consumption measured manometrically. Ethylene did not change respiratory rates in stems or leaves.

Leaves from 25-day-old plants were kept 12 hours in 10-liter vacuum desiccators containing 1:10 and 1:1000 ethylene-air mixtures. Leaf disks were then cut and respiration determined manometrically. The oxygen uptake by leaf disks from gassed leaves did not differ from the oxygen uptakes of disks from untreated controls. In 4 similar experiments, excised leaflets from 35-day-old tomato plants were gassed with 1:10, 1:100, 1:500, and 1:1000 ethylene-air mixtures. Two leaflets from each of 5 plants were used for each mixture. Controls were treated in the same way except that ethylene was omitted. Again, ethylene had no effect on leaf respiration.

Leaf disks from 35-day-old tomato plants were placed in ethylene solutions and vacuum infiltrated. The solutions were made from an ethylene solution saturated at 18°C and diluted 1:10, 1:100, and 1:1000. All contained M/30 potassium phosphate buffer at pH 5.9. After 1 hour in the solutions, leaf disks were removed, blotted dry, and quickly transferred to Warburg flasks. The ethylene solutions had no effect on oxygen uptake by the leaf disks.

Effect of ethylene on disease development.—Inoculated and noninoculated plants of resistant and susceptible tomato varieties were treated with ethylene to determine whether or not ethylene affects disease development as do ethanol (19) and several respiration inhibitors (10). Cuttings were allowed to take up bud cell suspensions by transpirational pull, and were placed the following day in solutions of ethylene prepared by diluting a saturated solution with tap water. The ethylene solutions were changed 1–2 times each day. Five plants were used for each treatment, and appropriate controls included inoculated untreated plants; noninoculated ethylene-treated plants; and noninoculated untreated plants. When cuttings developed roots, they were potted in sand and kept under conditions favoring disease. Fourteen days after inoculation, the plants were rated for severity of wilt and vascular browning.

Disease development was much faster in susceptible plants (var. Bonny Best) after 3 days of treatment with 1:10 or 1:100 ethylene solutions than in untreated inoculated controls. Ethylene-treated noninoculated control plants had temporary epinasty of leaves and temporary retardation of growth, but no other symptoms. Inoculated resistant plants (var. Jefferson) developed typical disease symptoms after a 6-day treatment with a 1:5 ethylene solution. Inoculated untreated controls had no disease symptoms. Noninoculated treated controls had epinasty and a slight yellowing of the lower leaves, and were slightly stunted at the end of the experiment. After 14 days, Fusarium was recovered by standard isolation procedures from the base to the top of the stems of inoculated ethylene-treated resistant cuttings, but was found only in the basal part of inoculated untreated resistant cuttings.

Effect of infection on cell permeability.—Conductivity measurements of leaching fluids from the leaves of healthy and diseased plants were made to determine whether infection affected tissue permeability. Data were taken at intervals as disease developed. In each experiment 30 inoculated plants and 30 noninoculated plants were used. An upper leaflet and a lower leaflet were taken from each plant, and 5 leaflets were placed in each flask containing 50 ml of double-glass-distilled water. Appropriate distilled-water controls were included. After 7 hours in the water the leaves were removed and the conductivity of the water was measured. Infection caused an increase in conductivity over that of healthy leaves, beginning 4 days after inoculation and increasing until advanced symptoms were appar-

ent (Fig. 1,D). This held true for both upper and lower leaves. Presumably this increase in conductivity was caused by an increased permeability of leaf cells to electrolytes. This experiment was repeated twice, with similar results.

DISCUSSION.—Comparisons of respiration in Fusarium-infected and healthy tomato plants showed that oxygen uptake and carbon-dioxide evolution were stimulated as early as 1 day after inoculation. Following this early, slight rise, respiration rates leveled out until about 9 days after inoculation, when another, and more striking, increase occurred, with disease-stimulated respiration reaching a peak shortly thereafter. The timing of this sequence and the degree of stimulation undoubtedly depend on environmental conditions. Also, lower leaves respond earlier than do upper leaves, with the stimulatory effects apparently moving up the plant as do the symptoms. The early response and the later, more marked, increase in respiration suggest that 2 reactions or series of reactions may be involved. The first may be the more sensitive, and is affected shortly after inoculation. When toxins become more concentrated, or when other toxins are formed, the second rise in respiration may occur.

The evidence indicates that a diffusible substance, which may be considered a respiratory toxin, is involved. Leaf tissue used in these experiments did not contain the pathogen. Thus, stimulated respiration probably resulted from a toxin or other stimulatory substance moving with the transpiration stream from the site of infection. Stem tissue also had stimulated respiration before the pathogen was present. One alternate theory should be considered: some material in the leaf that normally holds respiration in check is possibly moving toward the site of infection, becoming less concentrated in the leaf.

Ethylene, fusarinic acid, and pectic enzymes were immediately considered as possible respiration toxins. It is especially logical to suspect that ethylene is involved, since it is known to affect the respiration of certain fruits (7). Results of several types of experiments showed that ethylene did not affect leaf or stem respiration, which indicates that it is not the cause of respiratory disturbances in infected plants.

Pectic enzymes from the pathogen were not tested for respiratory effects, because of the probability of contamination with other metabolites that might have affected respiration. Instead, a commercial pectic-enzyme preparation was used, and results showed that such preparations did not affect respiration. However, many pectic-enzyme preparations should be used before this possibility is completely eliminated.

Fusarinic acid, a product of the tomato wilt Fusarium, was shown by Naef-Roth and Reusser (16) to be a respiratory inhibitor at concentrations of 10^{-4} M and above. The literature contains no reports of the effects of more dilute solutions on respiration. Relatively high levels of fusarinic acid were found to inhibit respiration, thus confirming the work of Naef-Roth and Reusser. Lower concentrations (10^{-5} to 10^{-8} M) had no effect on respiration. These experiments indicated that fusarinic acid was not responsible for observed respiratory disturbances in infected plants.

A careful distinction should be made between "wilt toxins" and the postulated respiratory toxins. Loss of turgidity apparently is associated with the action of pectic enzymes in xylem vessels (11). This is not to say that other toxins are not involved in the wilt diseases, but it is probably safe to say that these other materials do not directly bring about wilt. In the past, much confusion has centered on this point.

Significant increases in total nitrogen were found for plants in the advanced stages of disease, but the increases were not great enough to account for the increased respiration. The type of nitrogenous substance accumulating was not determined. Akazawa (3), working with black rot of sweet potato, found that sound tissue adjacent to infected areas had an increase in mitochondrial enzymes ("functional protein"). Also, a decrease in acid soluble nitrogen and an increase in acid insoluble nitrogen were reported (2). The possibility should be examined that nitrogen changes in Fusarium-infected plants may follow this pattern.

Certain observations indicate that the degree of respiratory increase in Fusarium-infected plants may depend on environmental conditions. In general the increase was less in winter than in summer, and more erratic. The reasons for this are not known, but temperature and light conditions are presumably involved. Light would seem to have both direct and indirect effects, since plants growing in low light intensity have few roots and develop new ones slowly. In plants with very weak root systems, systemic invasion by the fungus is sometimes retarded. Also, certain nutritional factors undoubtedly affect the respiratory response. These subjects will be considered in later work.

Permeability changes were measured in the present work by conductivity tests of leaching fluids from diseased and healthy plants. Permeability of leaf blade cells was increased as early as 4 days after inoculation, and continued to rise until advanced symptoms appeared. Changes in permeability may parallel the respiratory changes, since any change in respiration may affect permeability, and vice versa. The changes in leaf cell permeability give further support to the theory of systemic toxemia.

Dimond and Waggoner (8) have shown that ethylene is produced in diseased plants and causes epinasty, a symptom of Fusarium wilt of tomato. In the present study the observations of Dimond and Waggoner were confirmed and other effects of ethylene were noted. Disease development was more rapid in inoculated wilt-susceptible cuttings treated with ethylene than in untreated controls. Ethylene caused inoculated resistant plants to develop typical Fusarium wilt symptoms. This observation was strengthened when the causal organism was isolated from the base

to the tip of stems of inoculated treated resistant plants, while it was found only in the base of inoculated untreated resistant plants. Other substances known to alter resistance are sodium diethyl dithiocarbamate, thiourea, sodium fluoride, 2,4-dinitrophenol (10), and ethanol (19). How ethylene acts to break resistance is not known, but both it and ethanol are of special interest because both are produced by Fusarium.

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THE ASSOCIATION OF PRATYLENCHUS HEXINCISUS WITH CHARCOAL ROT OF SORGHUM [1]

Don C. Norton

SUMMARY

Greenhouse experiments indicate that part of the damage in Texas often attributed to *Macrophomina phaseoli* is due to the activity of *Pratylenchus hexincisus*. These two incitants apparently act independently. The effects of *M. phaseoli* and *P. hexincisus* on sorghum were significantly greater when the plants were under experimental drouth conditions than when not. *P. hexincisus* was found to lie, as a rule, parallel to the longitudinal axis of the roots, and always outside the vascular elements.

Charcoal rot, caused by the fungus *Macrophomina phaseoli* (Maubl.) Ashby, was a serious sorghum disease during the recent drouth years in Texas. A general survey for plant-parasitic nematodes, initiated in 1954, revealed that members of the genera Pratylenchus, Xiphinema, Trichodorus, and Tylenchorhynchus were frequently associated with the roots of sorghum infected with charcoal rot. In some cases, plants made normal growth for most of the season, but, as they approached maturity, which was often during the dry period, typical symptoms of charcoal rot appeared. In other instances, the most noticeable symptom was a failure of the head to fill out. In view of preliminary findings, it was thought desirable to make a re-evaluation of the disease with nematodes taken into consideration.

Materials and methods.—Abilene fine sandy loam soil infested with *Pratylenchus hexincisus* Taylor and Jenkins, *Tylenchorhynchus acutus* Allen, *Macrophomina phaseoli*, and *Gibberella fujikuroi* (Saw.) Wr. was used in the experiments. The soil was placed in 2-gallon crocks, steam sterilized, and divided into 3 groups. No further immediate treatment was made to 1 group. To a second group, *M. phaseoli* was added by mixing 100 g of infested sorghum to 2 gallons of soil. The inoculum was prepared by culturing the fungus on

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sterilized pieces of chopped sorghum stalks. To the third group of crocks, unsterilized field soil was added to sterile soil in a ratio of 1:3. The crocks were placed on a center greenhouse bench, where each set was divided into two series—"wet" and "dry." On the tenth day after seeding with Combine 7078 sorghum, seedlings were thinned to 3 plants per crock. There were 24 plants per treatment in both the wet and dry series. All plants were kept adequately moist for the first 34 days to ensure good establishment. Differential watering was made from the 35th to the 110th day, after which the plants were harvested. The water in the wet series was reduced slightly, but not to the point of wilting. The plants in the dry series were watered at the onset of temporary wilting. One-half liter of water was added at a time, and daily watering records were maintained. Records of plant height, dry weight of the aerial portion of the plants, and yield were taken at harvest. Upon completion of the experiment, the soil was stirred and replanted, and the test repeated.

Since members of the genus Pratylenchus are known endoparasites and are common in sorghum fields, an experiment was performed to find the role, if any, of *P. hexincisus* in the incidence and severity of charcoal rot. Preliminary greenhouse experiments using selective fumigation, for control of nematodes or both nematodes and fungi, were at first encouraging, but a lack of complete differential kill allowed the nematode population to increase, causing erratic results. Field tests were inconclusive for similar reasons. Abilene fine sandy loam soil was placed in 2-gallon crocks, steam sterilized, and divided into a wet and dry series, as before. The most common soil saprophytes were isolated from naturally infested field soil by the dilution plate method, cultured on yeast extract solution, and added to all crocks. These organisms were *Aspergillus niger* van Tieghem, *A. terreus* Thom, *Penicillium janthinellum* Biaurge, *Trichoderma viride* Pers. ex. Fr., 2 bacteria, and 1 actinomycete. Free-living nematodes, mostly of the genera Cephalobus, Eucephalobus, and Rhabditis, gained entrance to all crocks by watering, with a resulting population build-up. Following the infestation with saprophytes, no further treatment was given the control set of crocks. To a second set, cultures of *M. phaseoli* were added, as previously described. To the third set, about 500 individuals of *P. hexincisus*, obtained by Young's (11) method, were added to each plant on the 12th day after seeding, at which time the seedlings were thinned to 3 per crock. To the last set of crocks, both *M. phaseoli* and *P. hexincisus* were added, as above. Watering was performed similarly to that in the other experiments, except that the dry series was kept more moist than before, to prevent *M. phaseoli* from causing severe damage to the plants. No data were taken during this first run, for differences among treatments did not appear great enough. However, core samplings did indicate that the nematodes were increasing satisfactorily. When the plants matured, the tops were discarded and the soil stirred and reseeded. The experiment was conducted similarly to previous tests using differential watering. Disease ratings, dry weight of the aerial portion, and nematode counts were recorded at harvest. Nematodes were separated from soil essentially by Christie and Perry's (1) method, and from roots by Young's (11) technique. The crocks were then reseeded and the experiment repeated.

Microscopic examinations were made of root tissue of field-grown plants and of plants in the greenhouse that had been inoculated with pure cultures of *P. hexincisus*. Free-hand sections were cut with a razor blade, stained with cotton blue and lactophenol, and destained with lacto-phenol. Fine rootlets were stained whole.

Results.—In the experiment where sorghum was grown in sterile soil, soil infested with *M. phaseoli*, or field soil, significant differences between treatments were obtained in most cases (Table 1). There was no significant difference in height between plants grown in infested soil and field soil in the wet series, but dry weights were significant. Culms of plants grown in field soil were much thinner than those grown in infested soil. Part of the damage could be due to *G. fujikuroi*, but preliminary fumigation tests indicated that at least part was due to either *P. hexincisus* or *T. acutus*. Experimental drouth greatly reduced plant growth. Similar results were obtained when the experiment was repeated, except that, in the wet series, there was no significant difference in plant height although differences in dry weights were still evident. The phenomenon of height reduction upon repeated planting in sterile soil was noted on several occasions, although stalk rot did not occur after 2 replantings.

Table 1.—*Height, dry weight, and yield of plants at harvest of sorghum plants under various treatments (all values on a per plant basis)*

Treatment	Height		Dry weight of tops		Yield		Average water added in days 35–110	
	Wet series (cm)	Dry series (cm)	Wet series (g)	Dry series (g)	Wet series (g)	Dry series (g)	Wet series (liters)	Dry series (liters)
Sterile soil	98.3	64.0	36.0	24.8	13.4	5.2	6.5	4.6
Sterile soil + *M. phaseoli*	61.5	51.8	24.4	18.9	8.1	5.6	4.7	3.5
Field soil	64.3	42.0	18.1	12.8	5.8	2.8	3.8	2.9
$LSD_{.05}$	17.4	8.7	4.6	4.3	1.3	1.5		
$LSD_{.01}$	25.3	12.6	6.6	6.1	1.8	1.7		

TABLE 2.—*Disease rating, dry weight of tops, and nematode counts in various sorghum treatments*

Treatment[a]	Disease rating[b]		Dry weight of tops		Nematode counts at completion of experiment							
					Soil (½ pint)				Roots (1 gram)			
					Wet series		Dry series		Wet series		Dry series	
	Wet series	Dry series	Wet series (g)	Dry series (g)	*P. hexincisus*	Others	*P. hexincisus*	Others	*P. hexincisus*	Others	*P. hexincisus*	Others
Sterile soil	0.0	0.0	24.0	17.4	0	1380	0	1303	0	0	0	20
Sterile soil + *M. phaseoli*	0.0	2.1	20.8	14.1	0	2027	0	1370	0	10	0	30
Sterile soil + *P. hexincisus*	0.8	0.9	19.0	13.3	157	1430	493	1373	440	20	493	37
Sterile soil + *M. phaseoli* + *P. hexincisus*	1.1	3.7	20.4	10.7	265	1315	883	1854	620	20	533	150
$LSD_{.05}$	0.9	1.5	NSD	2.1								
$LSD_{.01}$	NSD	2.2	NSD	3.0								

[a] Soil saprophytes added to all cultures.
[b] 0–5 (no stalk rot—severe stalk rot).

Table 2 presents results of the experiments where cultures of *M. phaseoli* and *P. hexincisus* were added to soil, singly or in combinations. In well-watered plants, there was little significant difference in disease ratings or top weights. In the dry series, the highest disease rating as well as the lowest top dry weight were obtained when *M. phaseoli* and *P. hexincisus* were mixed. Lodging occurred only when both the nematode and fungus were present. Reduction in dry weight was about the same, whether nematodes or fungi were active, but reduction was about twice as much when both were mixed. This would indicate an independent relationship. Comparable results were obtained when the experiment was repeated.

Microscopic examination of the roots revealed that *P. hexincisus* invaded the rootlets abundantly, and for the most part were oriented parallel to the longitudinal axis of the root (Fig. 1). Great difficulty was experienced in locating nematodes in the old roots, but they were found with ease in rootlets. In all cases, nematodes were observed only in the cortical parenchyma. Graham (2) noted a similar situation existing in corn. Considerable sloughing off of the cortical tissues was observed in young roots. *M. phaseoli* invaded rootlets of healthy-appearing plants under no water stress and in the absence of *P. hexincisus*, but appeared unable to colonize the roots extensively.

DISCUSSION.—Lack of disease where *M. phaseoli* or *P. hexincisus* were not active would indicate that these are the primary incitants, with the other organisms playing a secondary although perhaps a contributing role.

There have been many attempts to correlate charcoal rot incidence with varying environmental conditions governing behavior of both host and parasite. Littauer (3) and Thirumalachar (8) have suggested that the greater incidence of the disease during hot, dry weather might be due to the greater growth of the fungus through the soil at higher temperatures, as compared with many soil borne parasites. Most temperature studies have been made on agar (4, 9, 12) although similar temperature data have been obtained

FIG. 1. *Pratylenchus hexincisus* inside sorghum rootlets.

in soil (5). Another opinion is based on the host's losing its ability to resist the fungus in hot, dry weather (10).

The writer, from experience with guayule, feels that drouth and its accompanying effects are more important to disease incidence than growth of the fungus through the soil. This is based on 1) the observation that the fungus makes little growth through the soil (5, 6); 2) the widespread presence of the fungus in Texas soils; 3) the observation that guayule plants have remained alive 4 years or more even when part of the plant was killed by charcoal rot, a similar observation also being made by Presley (7); and 4) the observation that progress of the disease in individual guayule plants ceases with the advent of rain or cool weather, with the plant surviving several more years, indicating the importance of host reaction. The relation of drouth conditions to charcoal rot incidence was emphasized when microscopic examinations showed that *M. phaseoli* invaded sorghum not under water stress, but was unable to make extensive colonization. The fact that *P. hexincisus* does not affect the severity of charcoal rot may be due to its inability to interfere with water translocation: it does not invade the vascular tissue. The results also emphasize, at least in this instance, the greater expression of nematode damage under drouth than under nondrouth conditions.

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UREDIOSPORE GERMTUBE FUSIONS IN PUCCINIA GRAMINIS [1]

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SUMMARY

Anastomoses between the germtubes of urediospores of 9 isolates of *Puccinia graminis* were found on 7 different media, which included artificial substrates and host surfaces. Germtubes of *P. graminis* var. *tritici* anastomosed with those of 4 other varieties of *P. graminis*. Mineral oil permitted spore germination but reduced anastomosis and entirely suppressed the formation of fusion bodies. Higher osmotic concentrations of the agar medium appeared to stimulate fusion body production. Nuclei were seen within fusion bodies, indicating that they may pass out of the germ tube in which they originate.

Since 1954, genetic evidence has been presented (3, 5, 9, 10, 14, 15) suggesting that nuclear exchanges between vegetative structures occur during the uredial stage of rust fungi. Since 1889, anastomosis of germtubes, appressoria, substomatal vesicles, or hyphae has been observed occasionally in at least 9 species of Puccinia (1, 2, 4, 6, 9, 12, 13).

The work reported herein was done (a) to find a medium that would greatly increase the numbers of anastomoses, (b) to learn whether germtube anastomosis is common when rust isolates (including several varieties of *Puccinia graminis* Pers.) are studied alone and as mixtures, and (c) to learn whether nuclei pass through the fusion tube. A preliminary report has been given (16).

Methods and results.—Freshly collected urediospores of *P. graminis* were employed throughout these studies. Usually the urediospores were germinated on a nutrient agar (13), but surfaces of wheat seedlings, water agar, mineral oil (Squibb mineral oil, heavy Californian liquid petrolatum), and agar containing varying amounts of sucrose, glucose, or sodium chlo-

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ride were also used. The amount of sugars or salt added to the medium was that suggested by Morse (8) and Meyer and Anderson (7). The moisture needed for spore germination in mineral oil was provided by incubating the urediospores in Petri dishes lined with moist filter paper. Several trials were made to determine the frequency of fusions on inoculated wheat seedlings kept overnight in dew chambers at room temperatures. Developing germtubes were examined on epidermal peels or in a manner similar to that described by Peterson (11).

Generally the spores were allowed to germinate, and the germtubes were examined by microscope after growing overnight at 15°–25°C. Since it is difficult to distinguish isolates of the stem rust fungus by urediospore size and shape, isolates with orange, red, and gray-brown urediospores were examined with a phase microscope. With this instrument, orange spores appeared colorless, and red or gray-brown spores appeared tan. Thus the phase microscope is particularly valuable for detecting fusions between germtubes of different-colored isolates in mixtures. Another method for determining racial identity of germtubes involved staining them with acid fuchsin, which stains orange urediospores pink, and gray-brown or red ones deep red.

Urediospores of *Melampsora lini* (Pers.) Lev., *P. graminis* Pers. var. *avenae* (Eriks. & E. Henn.) Seym., *P. graminis* Pers. var. *agrostis* (Eriks. & E. Henn.) Seym., and 10 isolates of *P. graminis* Pers. var. *tritici* (Eriks. & E. Henn.) Shear, Ball, Jacks., & Stakman were dusted separately onto nutrient agar. With each rust isolate tested the resulting germtube fusions and fusion bodies were so numerous that the germtubes were connected into a nearly continuous network. The contents of most fusion bodies appeared homogeneous (Fig. 1,A) and may have permitted protoplasmic exchange between the germtubes.

Urediospores of *P. graminis* Pers. var. *secalis* (Eriks. & E. Henn.) Seym. and of *P. graminis* var. *tritici* races 11 and 15B were sown separately on nutrient agar on slides and examined with the phase microscope. Many germtube fusions were seen. Attempts to trace the germtubes to the urediospores from the point of fusion were rarely successful: 18 distinct fusions were seen in race 11, 11 in race 15B, and 8 in *P. graminis* var. *secalis*. Fusion bodies were seen frequently but not counted.

It seemed possible that osmotic concentration of the medium might stimulate the formation of fusion bodies, for increased sugar concentration of the medium has been shown to stimulate formation of fusion bodies (13). Sucrose, glucose, or sodium chloride were added to water agar so that the osmotic concentration of the medium was about 5–7.5 atmospheres. Water agar and nutrient agar (13) without glucose were controls. In 3 tests, urediospores of wheat stem rust race 29 were dusted onto the various media; in one test, race GB2[3] was added. The fusion bodies in 3 fields of view (100 ×) per plate were counted and averaged. Since the results of each trial followed the same general trend, only 1 trial is presented in Table 1.

All three substances stimulated production of fusion bodies. When race 29 was tested, sodium chloride was not as effective as glucose or sucrose in permitting fusion bodies to form, but with race GB2 the salt was as effective as either sugar. In 1 trial a very few scattered fusion bodies were seen on the water agar controls.

Fusion of vegetative structures between isolates that are not genetically alike could presumably yield progeny genetically unlike the parents. Mixtures of races of *P. graminis* var. *tritici* were made on nutrient agar; race 11 (orange urediospores) was mixed with race GB2 (gray-brown urediospores) or with race 29 (red urediospores). Just before the preparations were examined they were stained with acid fuchsin. Many germtubes from spores of the different races in the mixture anastomosed, and fusion bodies also were seen frequently. Other mixtures of races of *P. graminis* var. *tritici* with different-colored urediospores were examined with the phase microscope, and the germtubes were traced from the fusion to the urediospores. Eight fusions between race 11 and race 15B on agar were seen. When orange urediospores of race 11 and gray-brown spores of race 121 were mixed in mineral oil, 1 fusion between the 2 races was seen.

Red urediospores of the *P. graminis* varieties *avenae*, *agrostis*, *secalis*, and *poae* (Eriks. & E. Henn.) Seym. were mixed separately with orange urediospores of race 11 of variety *tritici* on nutrient agar and in mineral oil. On agar, mixtures of variety *tritici* with varieties *agrostis* and *avenae* were examined after staining with acid fuchsin; mixtures of *tritici* and *secalis* and all mixtures in mineral oil were examined with the phase microscope. In each mixture, fusion was seen of germtubes of the several varieties (Table 2). Fig. 1,B illustrates a fusion between germtubes of the varieties *secalis* and *tritici*. The number of fusions recorded was small because only those fusions were

TABLE 1.—*The effect of sugar or salt in water agar on the production of fusion bodies in 2 races of P. graminis var. tritici*

Agar medium	Approx. osmotic concentration (atmospheres)[a]	Average number of fusion bodies[b]	
		Race 29	GB2
Glucose	5	20	15
Sucrose	5	15	20
Sodium chloride	5	10	35
Glucose	7.5	10	25
Sucrose	7.5	25	25
Sodium chloride	7.5	5	20
Water agar		0	0
Nutrient agar with glucose[c]		trace	trace

[a] Sucrose and glucose values from Morse. Sodium chloride values from Meyer and Anderson.
[b] Averages of 3 fields of view (100 ×).
[c] After Rodenhiser and Hurd-Karrer.

[3] GB2 is an undesignated race of *P. graminis* var. *tritici* with gray-brown urediospores.

counted that involved germtubes that could be definitely traced to the originating urediospores. On agar, many other anastomoses and fusion bodies were seen. In mineral oil, fusions were rare, but those that occurred could be definitely traced and established as real fusions. No fusion bodies were seen in mineral oil.

Many fusions of germtubes were observed on Khapli emmer and Little Club seedlings inoculated with urediospore mixtures of orange race 11 and race GB2 or with 15B alone. Fusion bodies were not numerous, but fused appressoria were seen. Germtube fusions were numerous. Fig. 1.C illustrates fusion of germtubes of race 15B on a seedling leaf of Little Club.

Germtubes and fusion bodies, on agar, were fixed in formalinacetic acid-alcohol or in formalin-proprionic acid-alcohol, and stained with Heidenhain's hematoxylin. Cytoplasm, which usually occupied the entire volume of the germtube, was highly vacuolate in the area adjacent to the spore and was relatively dense near the growing tip or cross walls. The nuclei occurred singly, at irregular intervals, 2–11 per germtube, averaging 6 each for 50 germtubes. Divisions occurred singly and not as conjugate nuclear divisions. Only one mitotic figure per germtube was seen, and spindles were not aligned in any particular plane.

The cytoplasm also contained numerous inclusions in addition to nuclei. In living material, studied with the phase microscope, many of these inclusions appeared to be mitochondria-like bodies; in stained preparations, cell constituents other than the nuclei absorbed the stain, often obscuring other structures

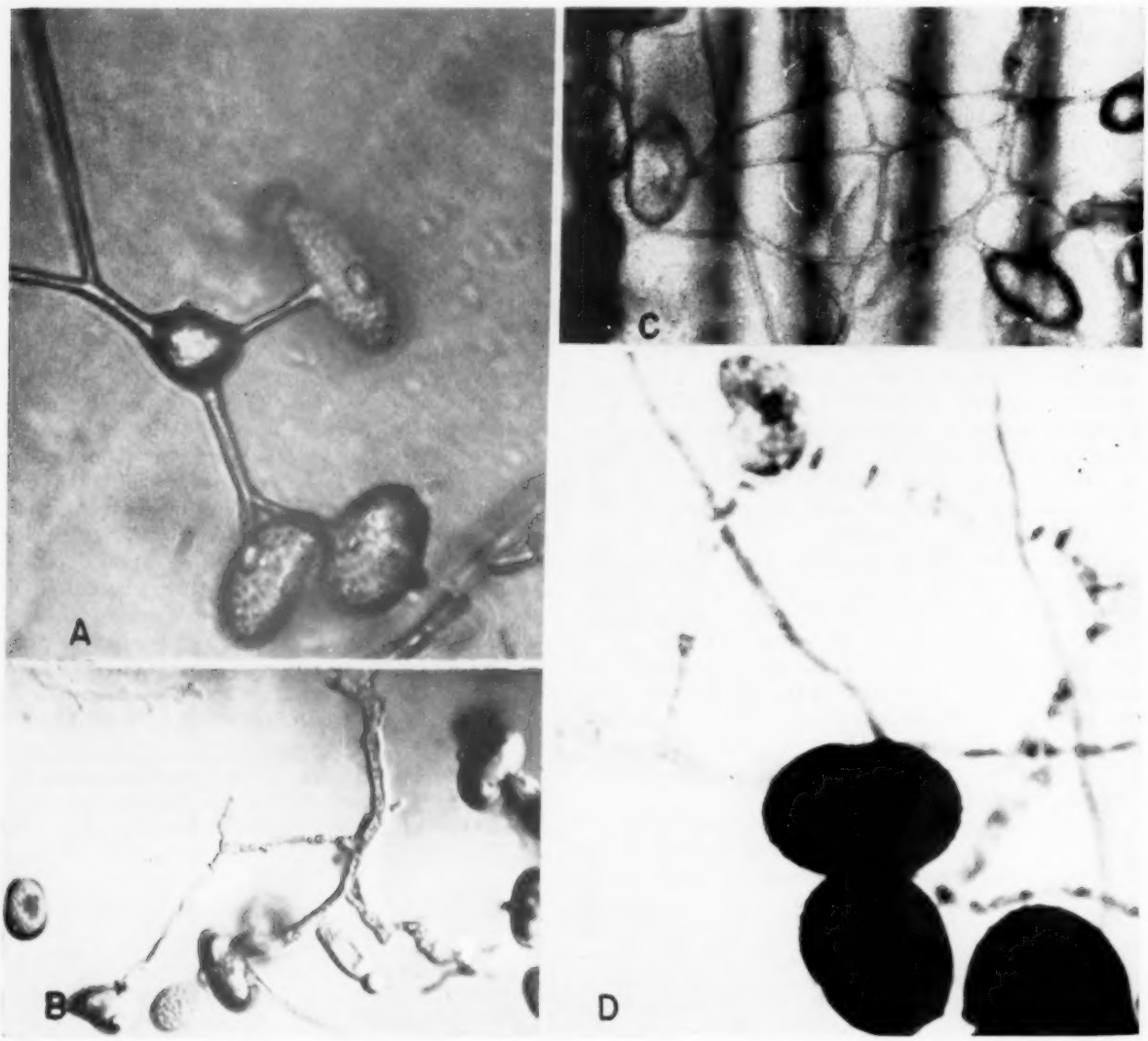

FIG. 1. A) Germtube fusions and a fusion body involving 3 urediospores of *P. graminis* var. *tritici* on nutrient agar. B) Fusion of germtubes between *P. graminis* var. *tritici* and *P. graminis* var. *secalis* in mineral oil. C) Network of fused urediospore germtubes of race 15B of wheat stem rust on Little Club seedling leaves. D) A pair of nuclei in a fusion body, formed by germtubes from 2 urediospores of *P. graminis* var. *tritici*.

TABLE 2.—*Numbers of germtube fusions between varieties of P. graminis when urediospores were germinated on nutrient agar or in mineral oil*

Mixtures of varieties	Number of fusions
On nutrient agar[a]	
tritici × avenae	1
tritici × agrostis	2
tritici × secalis	10
In mineral oil[b]	
tritici × avenae	3
tritici × secalis	8
tritici × poae	2

[a] After Rodenhiser and Hurd-Karrer.
[b] Squibb Mineral Oil, heavy Californian liquid petrolatum.

completely.

In fusion bodies, 2–4 nuclei were commonly seen along with protoplasmic materials that apparently had been moving between germtubes and the fusion body. A fusion body containing 2 nuclei lying close together is seen in Fig. 1.D.

DISCUSSION AND CONCLUSIONS.—Anastomosis among germtubes arising from urediospores of *P. graminis* is common. They have been seen on the surfaces of artificial media, in mineral oil, and on the surfaces of wheat leaves. The medium influences the type and frequency of anastomosis. In mineral oil, fusion bodies did not form; on wheat seedling leaves they formed only occasionally; on water agar to which sugars or salt were added the number of fusion bodies was greatly increased. Only a few germtubes anastomosed in mineral oil, but on agar media and host leaves, germtube anastomosis was very common. It is probable that, under natural conditions, fusion bodies do not play an important role in the exchange of protoplasmic materials between germtubes, for they were rarely found on plant surfaces.

The random arrangement of nuclei in germtubes confirms similar reports made in the past. The discovery of nuclei within fusion bodies clearly indicates that they may pass out of the germ tubes in which they originate. Thus nuclei from different sources may be brought together to form new combinations. The fusion of germtubes between the several varieties of *P. graminis* is particularly interesting in this respect, for it indicates that nuclear material from widely different sources may be brought together during the uredial stages. This process may be very important in producing new biotypes within species of rust fungi, especially those species whose alternate hosts are not commonly infected.

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THE INHIBITION OF THE INFECTIVITY OF TOBACCO MOSAIC VIRUS BY SOME SYNTHETIC AND NATURAL POLYELECTROLYTES [1]

Mark A. Stahmann and S. S. Gothoskar [2]

SUMMARY

Polyglutamic acid, polyacrylic acid, and sodium polypectate, when mixed with tobacco mosaic virus, reduced virus infectivity. Infectivity was reduced when polyglutamic acid was applied before the virus inoculum but not after, which suggests that these compounds act on the plant cells rather than on the virus. *Trichothecium* culture filtrates reduced virus infectivity by an effect on the host cells. The fact that the inhibitory action of culture filtrates was reduced markedly by treatment with a weak anion exchange resin indicates that the activity of the culture filtrate was similar to that of the acidic polyglutamic acid or of sodium polypectate. It is postulated that such acidic polyelectrolytes combine with or block basic receptor sites of susceptible host cells.

A wide variety of substances, when applied to plants in admixture with tobacco mosaic virus, have been shown to inhibit infection by the virus. This reduction in infectivity is believed in some cases to be due to combination of the material with the virus, whereas in other cases the inhibitor acts on the cells of the host and has no direct effect on the virus. The pertinent literature on the subject has been reviewed by Gupta and Price (6) and by Bawden (1).

To date, most of the work on virus inhibition has been with plant extracts or with fungal culture filtrates. The chief disadvantage of such materials is that they contain a wide variety of substances of an unknown nature, many of which inhibit infection by viruses and consequently make interpretation of results difficult. The work presented here is an investigation into the mode of inhibition of tobacco mosaic virus infectivity by certain acidic synthetic polypeptides, polysaccharides, and synthetic polymers. These substances may be structural models of some of the virus inhibitors found in nature and, since their properties and chemical structure are known, provide a better basis for interpretation of results.

Stahmann et al (9) reported the inhibition of tobacco mosaic virus by lysine polypeptides. They suggested that the reduction in infectivity was due to a complex formation involving ionic bonds between the acidic groups of the virus and the basic epsilon amino groups of the lysine polypeptide. Burger and Stahmann (2) studied the interaction of lysine polypeptides with tobacco mosaic virus *in vitro* and observed that lysine polypeptides precipitated tobacco mosaic virus at pH levels at which the virus and the polypeptide were oppositely charged. The inhibitory action of polylysine was therefore attributed to its effect on the virus and not on the susceptibility of the host. It was of interest to study the effect of acidic polyelectrolytes such as polyglutamic acid, polyacrylic acid, pectic acid, and related substances on infection by tobacco mosaic virus.

MATERIAL AND METHODS.—The tobacco mosaic virus used in these experiments was obtained from infected field-grown tobacco plants according to the method of Stanley (10). The preparations were viscous, opalescent concentrates containing 29 mg virus per ml, based on Kjeldahl nitrogen. Poly-L-glutamic acid (4), with an average chain length of 129 glutamic acid residues per molecule, was prepared by Dr. Maurice Green. The polypeptide was suspended in water and the pH adjusted to 7.0 by the addition of sodium hydroxide, whereupon the polypeptide dissolved. Assays for virus activity were carried out by the method of Kuntz and Walker (8), based on the local-lesion response of *Nicotiana glutinosa*. The hybrid *N. glutinosa* × *N. tabacum* was also used. The test and control solutions were spread with glass spatulas on the left and right half-leaves, respectively. Carborundum (500 mesh) was sprinkled evenly on the leaf surface before inoculation. A Latin square arrangement of inoculated leaves minimized variation in number of lesions due to individual plant and leaf variability. Local lesions usually were counted 3–5 days after inoculation, depending on temperature and light conditions. Percentage inhibition was calculated as the difference between the number of lesions from the test sample and that of the control, expressed as a percentage of the number of lesions of the control.

EXPERIMENTAL RESULTS.—In a preliminary experiment on the effect of polyglutamic acid on virus infection, different concentrations of the polypeptide were mixed with a solution of 2.9 μg of the virus per ml of 0.1 M phosphate buffer (pH 7), and the mixture rubbed on half-leaves. The other half of each leaf was inoculated with the same concentration of the virus in phosphate buffer. The polypeptide caused a marked reduction in virus activity (Table 1). A similar experiment with L-glutamic acid verified that this reduction was not due to the free amino acid, but rather to the polypeptide.

Inhibition studies indicated that 2 widely different acidic polyelectrolytes inhibited virus infectivity. Polyacrylic acid (Monsanto), with an average chain length of 15,000 units, and sodium polypectate (Or-

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TABLE 1.—*Relative infectivities of mixtures of tobacco mosaic virus alone and when mixed with acidic polyelectrolytes, as indicated by numbers of lesions on inoculated half-leaves of a tobacco hybrid*[a]

Polyelectrolyte		Local lesions, 4 half-leaves		
Kind	Conc. mg/ml	Mixture	Virus alone	Inhibition[b] (%)
L-Polyglutamic acid	33	104	766	86
L-Polyglutamic acid	10	130	744	80
L-Polyglutamic acid	5	200	730	73
L-Polyglutamic acid	1	514	724	29
L-Glutamic acid	33	419	433	4
L-Glutamic acid	10	280	160	0
L-Glutamic acid	5	284	328	0
Polyacrylic acid	33	43	583	93
Polyacrylic acid	10	53	549	90
Polyacrylic acid	5	74	487	85
Polyacrylic acid	1	92	422	78
Sodium polypectate	2.5	216	857	75
Sodium polypectate	1.0	324	891	63
Sodium polypectate	0.5	321	861	63

[a] All inocula contained 2.9 μg virus per ml in 0.1 M phosphate buffer at pH 7.

[b] Difference in number of lesions produced by the 2 inocula multiplied by 100 and divided by number of lesions produced by the virus alone.

ange Exchange Products Company) also had a marked effect on virus activity.

Lysine polypeptides inhibit tobacco mosaic virus infection when the polypeptides and the virus are mixed together at pH levels at which they are oppositely charged. Burger (3) observed that application of the lysine polypeptide followed by the virus, or vice versa, did not reduce infectivity of the virus; it was only when the 2 were mixed together that infectivity was reduced. He concluded that the effect of the basic lysine polypeptide was on the virus, not on the plant.

The experiments with polyglutamic acid were conducted at pH 7. At this pH both the virus and the polyglutamic acid are negatively charged, and reduction in infectivity could not be due to a combination of the two substances, but to the effect of the acidic polypeptide on the plant.

To test this hypothesis further, the leaves of 1 group of plants were rubbed with a 0.1 ml solution of polyglutamic acid (33 mg per ml), washed immediately with distilled water, allowed to dry, and then inoculated with the virus (2.9 μg per ml). Leaves of a second group of plants were similarly rubbed with the polypeptide solution, which was allowed to remain on the leaves for 1 hour before continuation of the treatment. On the third group of plants the virus solution was applied first, rinsed off immediately, and the leaves allowed to dry. The polyglutamic acid was then applied, allowed to stand for 1 hour, after which the leaves were washed and allowed to dry. All virus and polyglutamic acid applications were made in the presence of Carborundum.

A 30% decrease in infectivity occurred when the polypeptide was applied to the plants and allowed to remain for 1 hour (Table 2). When the polypeptide

TABLE 2.—*Relative amounts of inhibition of tobacco mosaic virus by polyglutamic acid when the 2 were applied in different orders to half-leaves of a tobacco hybrid*

Treatment[a]					
Material applied first	Hr. between applications[b]	No. of leaves	No. of lesions Control	No. of lesions Test	Inhibition[c] (%)
Polyglutamic acid	0	11	1023	1015	0
Polyglutamic acid	1	10	1525	1077	30
Virus	0	11	1150	1020	0

[a] 0.1 ml of polyglutamic acid solution containing 33 mg/ml was applied by rubbing with carborundum; the virus inoculum contained 2.9 μg virus per ml in 0.1 M phosphate buffer at pH 7.

[b] Zero interval indicates that the material first applied was washed off the leaves immediately, and then the second material applied.

[c] Difference in number of lesions produced by the 2 inocula multiplied by 100 and divided by number of lesions produced by the virus alone.

was washed off immediately following application, or applied after the virus, no significant decrease in infectivity was obtained. The concept that the effect of the polyglutamic acid is principally on the plant is supported by these data and the need for a period of contact to induce marked inhibition.

It was of interest to determine whether an increase in time of contact beyond 1 hour would result in a greater inhibition of infectivity. When the polypeptide was in contact with the leaf surface for 2, 3, or 4 hours and then washed off, and the plants inoculated with the virus, no significant increase in inhibition occurred over that attained after 1 hour.

Another method of determining whether inhibition results from an effect on the virus or on the host would be to determine the effect of the material on virus infectivity using different assay plants. If the inhibitor combines with the virus, no striking differences in the degree of inhibition among different host species would be expected. Therefore, some of the experiments that were performed on hybrids were repeated on *N. glutinosa*.

The inhibition of tobacco mosaic virus infectivity was greater on the hybrid than on *N. glutinosa* (Table 3). This is further evidence that the inhibition by polyglutamic acid is caused by an effect on the plant. The data are consistent with the view that acidic polyelectrolytes such as polyglutamic acid may reduce virus infectivity by blocking basic virus receptor sites of the leaf cells. This would seem to result from the formation of ionic bonds between the negatively charged inhibitor and the positively charged receptor sites. Since the negative charge on the polyglutamic molecule is due to dissociable carboxyl groups, esterification of the carboxyl groups should destroy the negative charge and reduce this combination. Unfortunately, the ester of polyglutamic acid is insoluble in water and cannot be used. The virus inhibitory activities of sodium polypectate and pectin were therefore compared. The sodium polypectate had a methoxyl content of 0.26 per cent, and relatively few of its carboxyl groups were esterified, whereas the

TABLE 3.—*Relative amount of inhibition by polyglutamic acid of infection by tobacco mosaic virus on plants of Nicotiana glutinosa and of a N. tabacum × N. glutinosa hybrid*

Treatment[a] in order of application	Concentration of inhibitor (mg/ml)	Inhibition[b] %	
		Hybrid	*N. glutinosa*
Inhibitor and virus mixed	33	86	66
Inhibitor and virus mixed	10	80	15
Inhibitor and virus mixed	5	73	9
Inhibitor and virus mixed	1	29	8
Polyglutamic acid first, virus immediately after	33	0	0
Polyglutamic acid first, virus 1 hour later	33	30	20

[a] The virus inoculum contained 2.9 μg virus per ml in 0.1 M phosphate buffer at pH 7.

[b] Difference in number of lesions produced by the 2 inocula on 12 leaves multiplied by 100 and divided by number of lesions produced by the virus alone.

pectin had a methoxyl content of 8.75 per cent, and was 50 per cent esterified. On a weight basis, sodium polypectate was 5 times as effective as pectin in inhibiting tobacco mosaic virus.

Gupta and Price (6) studied in some detail culture filtrates of *Trichothecium roseum* and concluded that their inhibitory activity was due to an alteration in host susceptibility. It was of interest to examine these culture filtrates for the presence of materials having properties similar to the ones tested by us. Culture filtrates of *T. roseum* were prepared by the method of Gupta and Price (5). These culture filtrates showed a marked reduction in virus infectivity when applied to the plant in admixture with the virus. The reduction was evident even when the filtrate was diluted 10-fold. When the culture filtrate (undiluted) was sprayed on the leaves and washed off immediately, and the leaves then inoculated with the virus, no reduction in infectivity was obtained. When the culture filtrate was allowed to remain on the leaves for 1 hour and then washed off before inoculation, an 80 per cent reduction in infectivity resulted. These results closely parallel those obtained with polyglutamic acid, suggesting that inhibition in both cases may be due to a similar mechanism. The report by Gupta and Price (5) that the agent present in *T. roseum* filtrates is heat-stable and non-dialyzable was confirmed. If the agent were a polysaccharide similar in nature to sodium polypectate, which contains a large number of carboxyl groups, then at pH 5 one would expect it to be negatively charged and consequently capable of being absorbed by a weak anion exchange resin. Accordingly, a small amount of Amberlite-IR-4B, a weak anion exchange resin, was buffered at pH 5 and shaken with 10 cc of culture filtrate. The resin was removed by centrifugation, and the supernatant tested for virus inhibitory activity. It was found to be much less potent than the starting material (Table 4). These results suggest that the inhibition of tobacco mosaic virus by *T. roseum* culture filtrates is in part attributable to the presence of acidic compounds. In this connection it is interesting to observe that Hirth and Drouhet (7) reported inhibition of tobacco mosaic virus by a capsular polysaccharide from *Torulopsis neoformans*. This polysaccharide inhibited tobacco mosaic virus more strongly than did starch, inulin, or glycogen, and differs in chemical composition in that it consists of xylose and mannose linked to an acidic uronic acid.

DISCUSSION.—We believe that the results obtained in this and many other studies of the action of virus inhibitors on tobacco mosaic virus can be best explained on the basis of an ionic combination of the inhibitor with either the virus particle or the virus-receptor sites of the plant cells. Either combination may block or prevent the initial binding of the virus by the host cell. The concept of a virus-cell attachment involving ionic or electrostatic forces is not new in the field of virology. Tolmach (11) has reviewed work that demonstrated that the initial union of bacterial viruses and host cells involves ionic groups at the surface of both the virus particle and the bacterial cell. Such reactions are very rapid. The fact that rinsing the plant leaves immediately after inoculation has little or no effect on subsequent virus infection suggests that a strong, very rapid, initial reaction must have occurred between the host cells and the virus particles. Slagel and Stahmann have observed, in unpublished experiments, that considerable tobacco mosaic virus is removed from solution by mixing with a suspension of washed tobacco pith cells.

We consider the results of studies with the ionic or polyelectrolyte inhibitors to be consistent with the concept of initial ionic interaction between tobacco mosaic virus and susceptible plant cells. Basic polyelectrolytes as the lysine polypeptides must inhibit tobacco mosaic virus by combining with the virus, not with the host plant. Acidic polyelectrolytes, such as polyglutamic acid, and the acidic polysaccharides seem to act on the cells of the host plant. They may slowly combine with the host cells in such a way as to block

TABLE 4.—*Inhibition of tobacco mosaic virus infection by culture filtrates of Trichothecium roseum before and after treatment with Amberlite IR-4B at pH 5*

Filtrate		Local lesions, 4 half-leaves of tobacco hybrid		Inhibition[b] (%)
Dilution	Kind	Mixture[a]	Virus alone[b]	
1:2	Untreated	35	1215	97
1:2	Treated	192	1683	64
1:4	Untreated	125	1478	92
1:4	Treated	275	504	45
1:8	Untreated	175	1418	88
1:8	Treated	340	367	7
1:10	Untreated	222	1158	81
1:10	Treated	328	306	0

[a] All inocula contained 14 μg virus per ml.

[b] Difference in number of lesions produced by the 2 inocula multiplied by 100 and divided by number of lesions produced by the virus alone.

or mask virus receptor sites, preventing their subsequent interaction with the virus.

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THE PSEUDOPLEA-STEMPHYLIUM COMPLEX ON ALFALFA [1]

W. C. McDonald [2]

SUMMARY

Cultural and pathogenic studies showed that *Stemphylium botryosum* and *Pseudoplea trifolii* were not taxonomically related. Isolates of *P. trifolii* from Manitoba, Minnesota, Wisconsin, and Pennsylvania were culturally similar on several media, and no conidia were observed under any conditions. Isolates of *S. botryosum* from Manitoba and New Hampshire produced mature ascospores of *Pleospora herbarum* on sterilized alfalfa leaves and on agar media. A method was developed of inoculating alfalfa with ascospores of *P. trifolii*. Mature ascospores were produced in 5–10 days by isolates grown on V–8 juice agar at 60°F in light. Typical Pseudoplea leaf spots developed within 48 hours after sporulating Petri-dish cultures were inverted over healthy detached alfalfa leaflets floating in distilled water or over mature plants in a humidity chamber.

There has been considerable confusion in the literature over its taxonomy and pathogenicity since the fungus that causes Pseudoplea leaf spot of alfalfa was first identified. Since then it has had a variety of names, depending on the host from which it was isolated, but, by 1953, *Pseudoplea trifolii* (Rostr.) Petr. was generally accepted as the name of this fungus, which also causes pepper spot of clovers. It is a homothallic ascomycete, and no conidial stage was reported before 1953. Until the publication of Smith's paper (14), in 1940, similar confusion existed with respect to the taxonomy of *Stemphylium botryosum* Wallr., the incitant of stem and leaf spotting of alfalfa and red clover. Smith and others have shown that its perfect stage is *Pleospora herbarum* (Pers. ex Fr.) Rab. In 1953, Nelson and Kernkamp (12) reported that several monoconidial isolates of *S. botryosum* produced perithecia of *P. trifolii* (syn. *P. briosiana* (Poll.) Hoehn.), both on artificial media and on the leaves of inoculated alfalfa plants, and, conversely, that single-ascospore cultures of *P. trifolii* developed conidia of *S. botryosum*. They also isolated *P. trifolii* in the spring, and *S. botryosum* later in the season, from the same type of spots on the leaves. These results led them to the conclusion that *P. trifolii* was the perfect stage of *S. botryosum*.

In the present study Nelson and Kernkamp's results were checked with isolates of Pseudoplea and Stemphylium from alfalfa, sweet clover, and red clover in Manitoba, and isolates received from Minnesota, Wisconsin, Pennsylvania, and New Hampshire.

CULTURAL STUDIES.—The cultures of *P. trifolii* originating from material collected in Manitoba were obtained either by plating pieces of surface-sterilized stem tissue containing black spots or by placing spotted leaves on moist filter paper in the bottoms of Petri dishes and isolating single ascospores that had been discharged upward onto water agar. All 8 ascospores from one ascus were often grouped so that they could be isolated separately. These cultures were similar to isolates received from F. R. Jones, of Wisconsin; A. H. Ellingboe, of Minnesota; and R. A. Kilpatrick, of New Hampshire.

S. botryosum was isolated by plating pieces of blackened stem tissue, or by transferring spores from a leaf incubated in a moist Petri dish to water agar

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plates, from which single spores were then transferred to agar slants. Cultures obtained from alfalfa leaves and red clover and sweet clover stems agreed culturally with an isolate from alfalfa sent by R. A. Kilpatrick. All isolates were maintained on either potato-sucrose agar or V-8 juice agar slants, and stock cultures were stored under mineral oil.

A method of producing ascospores from isolates of both fungi was of primary importance to the study. Most of the reports in the literature (5, 6, 8, 9) indicate that it is difficult to obtain mature perithecia of *P. trifolii* in culture; Nelson and Kernkamp (12) reported success on artificial medium, but did not state its composition. Similar difficulties have been experienced in attempts to produce *P. herbarum* on agar media (14). Mature ascospores of both *P. trifolii* and *P. herbarum* were first produced by the use of sterilized leaflets of alfalfa, as suggested by Miller (9). Leaflets, on filter paper covering a thin layer of moistened vermiculite in a Petri dish, were autoclaved ½ hour at 15 lb. pressure. Eleven isolates of *P. trifolii* and 4 of *S. botryosum* were transferred to the sterilized leaflets and incubated at 60°F in sunlight. In 15–19 days mature ascospores (Fig. 1,A,B) conforming to those described by Miles (8) were observed inside the lids of the Petri dishes of all 11 cultures of *P. trifolii*. In 7 weeks, 3 of the 4 cultures of *S. botryosum* produced mature ascospores of *P. herbarum* (Fig. 1,C) identical to those illustrated by Smith (14). The leaflets inoculated with *S. botryosum* were covered with conidia, but no conidia were ever observed in cultures of *P. trifolii*.

Reported benefits of V-8 juice agar on the sporulation of *S. botryosum* (10) suggested the use of this medium to induce cultures of *P. trifolii* to form conidia of *S. botryosum*. Single-spore cultures of 16 isolates of *P. trifolii* from Manitoba, Wisconsin, and Minnesota were incubated on V-8 juice agar at 60°F in diffuse sunlight. No conidia of any kind were observed in these cultures (Fig. 1,D), but ascospores were observed in varying abundance inside the lids over all isolates within 5–10 days. Single-spore cultures of 4 isolates of *S. botryosum* were also grown on V-8 juice agar (Fig. 1,E). Conidial production was better on V-8 than on potato-sucrose agar, and mature perithecia of *P. herbarum* were produced by all 4 isolates within 42 days. Equally good perithecial production in Pleospora was obtained by using oatmeal, cornmeal, and potato-sucrose agars under the same conditions.

PATHOGENICITY STUDIES.—*S. botryosum* is well recognized as a leaf and stem pathogen of alfalfa (2, 14, 15). The pathogenicity of *P. trifolii* on alfalfa is not so well documented, however. Two investigators were unable to obtain infection of healthy leaflets (7, 9), and Miles (8) was successful in obtaining infection only when leaflets bearing perithecia were suspended over the plants. Hopkins (5) also used this method in successfully inoculating white and red clover. Nelson and Kernkamp (12) reported infection on alfalfa from ascospores of *P. trifolii*, but did not say what medium the ascospores were produced on, or how the plants were inoculated.

As preliminary tests of the pathogenicity of Manitoba isolates of *P. trifolii* and *S. botryosum* from alfalfa, agar cultures were comminuted in a Waring Blendor, diluted with distilled water, and sprayed on 3-month-old alfalfa plants. The plants were incubated in a humidity chamber for 72 hours at 70°F. No symptoms appeared on the plants inoculated with *P. trifolii*, but typical leaf spotting and stem blackening occurred on alfalfa plants inoculated with *S. botryosum*.

When it was found that ascospores of *P. trifolii* could be produced on V-8 juice agar, Yarwood's (16) detached-leaf method was used to inoculate alfalfa. Fully expanded leaflets were floated on distilled water in Petri dishes, and sporulating cultures of *P. trifolii* were inverted over them for 24 hours. Within the next 24 hours, typical lesions of Pseudoplea leaf spot appeared on the leaflets. Ten isolates from Manitoba, 2 from Minnesota, and 1 each from Wisconsin and Pennsylvania were tested. Each proved pathogenic, with some differences in the amount of spotting. The differences were correlated with ability of the isolates to sporulate. Cultures on V-8 juice agar also proved successful in inoculating potted plants. Sporulating Petri-dish cultures were inverted on a wire mesh above alfalfa plants enclosed at 70°F in a plastic chamber. A high humidity was maintained by periodically spraying the inside of the chamber. Typical spots appeared within 72 hours and developed in the same manner as those on detached leaflets.

Symptoms agreed with those described in the literature (5, 6, 8, 9). The spots, at first black pin-point lesions, surrounded in many cases by a slight yellow halo, enlarged to form the typical sunken, gray-centered, reddish-brown-bordered spots. The individual lesions produced by the isolates tested did not attain diameters greater than about 1 mm. Leaflets on which spotting was profuse began to turn yellow after the fourth day. By the twelfth day, mature perithecia were numerous on white areas of the leaflets, where several spots had coalesced or where the leaflets had become watersoaked and decolorized. No perithecia were observed on spots that had not enlarged beyond 1-mm diameter and were surrounded by green tissue.

Examination of decolorized leaflets inoculated with Pseudoplea ascospores confirmed Miles' report (8) of direct penetration, and his description of the process. Ascospores germinated quickly and penetrated directly through the cells on which they fell. The epidermal cells were discolored, and, later, the palisade cells through which the mycelium ramified.

Plate cultures of *S. botryosum* were also used to inoculate detached leaflets, but symptom expression was erratic. Cultures producing ascospores of *P. herbarum* and younger cultures, with conidia only, were inverted over detached leaflets for 24 hours. A few tiny brown spots appeared on some leaflets 4 days later, but often no visible symptoms were apparent despite the presence of numerous germinating asco-

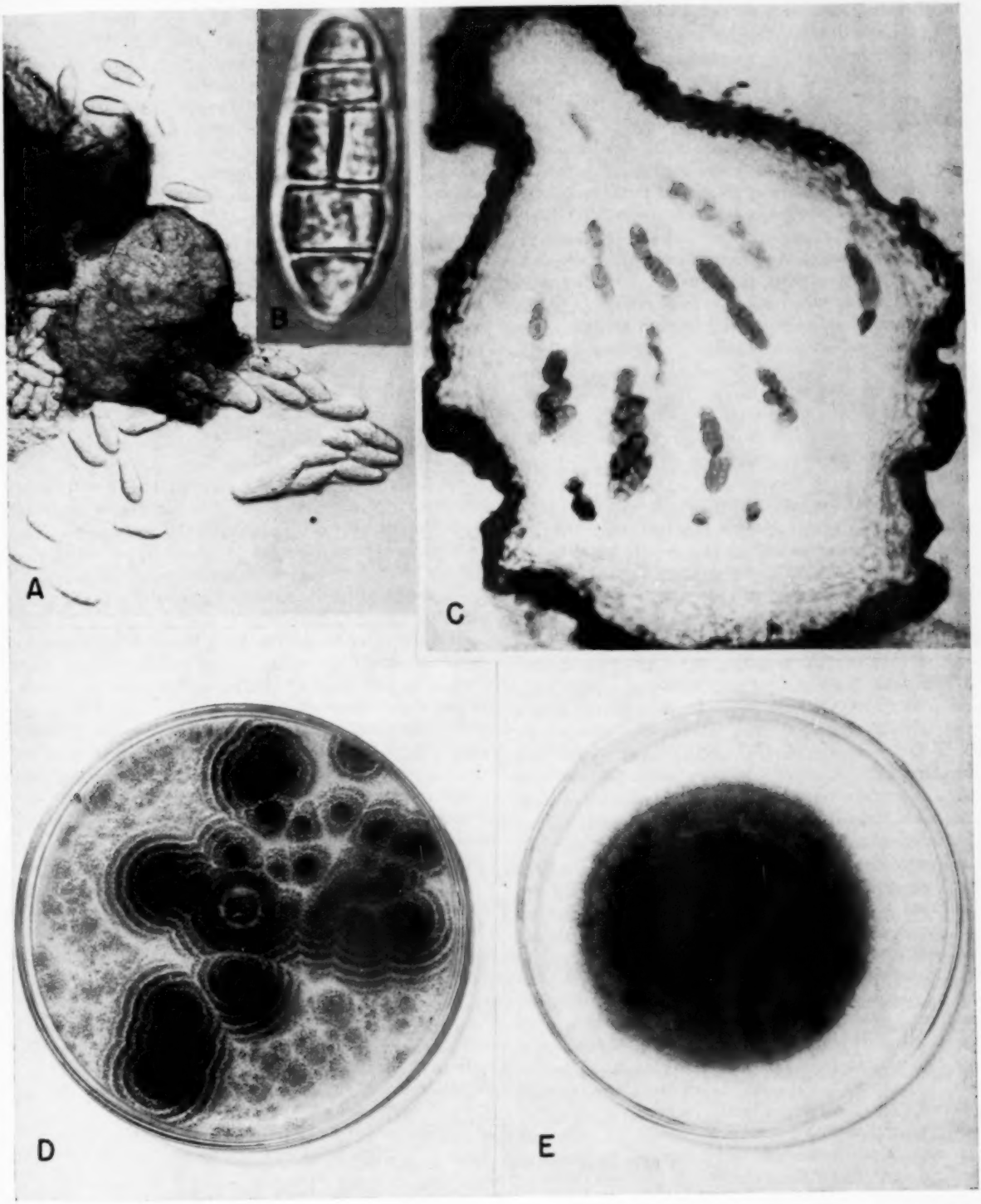

FIG. 1. A) Squashed perithecium, ascus, and ascospores of *Pseudoplea trifolii* (225×). B) Ascospore of *P. trifolii* showing gelatinous sheath (1250×). C) Cross section of a perithecium of *Pleospora herbarum* (300×). D,E) Growth and sporulation on V-8 juice agar after 11 days—of *P. trifolii* (D) and *Stemphylium botryosum* (E). Satellite colonies have developed from ascospores discharged from the central transplant of *P. trifolii*.

spores or conidia. Within 2 weeks the leaflets bearing spots became white and watersoaked and bore numerous conidia and a few immature perithecia of *P. herbarum*. Leaflets showing no symptoms at 4 days sometimes developed small white chlorotic areas in 2 weeks; more often they developed no definite spotting but gradually became watersoaked and covered with conidia. The typical white-centered, reddish-bordered spots that result from inoculating healthy plants with conidial suspensions seldom appeared on detached leaflets. On decolorized leaflets examined 48 hours after inoculation, 95 of 100 ascospores observed had produced fine germ tubes, growing at random over the surface of the leaf and finally entering through stomata. The other 5 had formed swollen cells on the surface and penetrated directly, discoloring the host cells beneath. After entering the sub-stomatal cavity or epidermal cell, the germ tubes enlarged and branched profusely. These branches invaded surrounding palisade cells and caused discoloration. Germinating conidia behaved in the same manner. This method of penetration by *S. botryosum* has been described by Smith (14) and Nelson (11).

Association of the fungi with the disease.—Nelson and Kernkamp (12) reported that both *P. trifolii* and *S. botryosum* could be isolated from the same spot, and used this information in establishing the relationship between the two. They reported that only *P. trifolii* was obtained in isolations from field material early in the season, whereas later on Stemphylium only was isolated from the same type of spot. In Manitoba, both conidia of *S. botryosum* and perithecia of *P. trifolii* have often been observed on the same alfalfa leaf spot even late in the season. To check their compatibility, detached leaflets were first inoculated with *P. trifolii*, by inverting a plate culture over the leaflets for 24 hours, and inoculated 4 days later with *S. botryosum*, by tapping a conidial colony inverted over the floating leaves. Another set of floating leaves was inoculated in the reverse order. Two other sets were inoculated with each singly. All were incubated 15 days at 60°F in diffuse sunlight. After that period the leaflets inoculated with both organisms bore mature perithecia of *P. trifolii*, numerous conidia of *S. botryosum*, and immature perithecia of *P. herbarum*, all intermingled in the dead areas. Leaflets inoculated only with *P. trifolii* had Pseudoplea perithecia only; leaves inoculated only with *S. botryosum* had conidia and immature perithecia of *P. herbarum* only. Apparently, each of these fungi can readily colonize a lesion produced by the other. This situation, plus the similarity of primary symptoms, on both leaf and stem, induced by each singly, makes diagnosis in the field very difficult.

Discussion.—Alfalfa breeders are becoming increasingly concerned with the problems involved in breeding for resistance to foliar diseases. The symptoms of at least 5 of these diseases are very similar, often making differentiation difficult under field conditions. Reports that 2 or more of the fungi associated with the various diseases are in fact different stages of the same organism should be adequately confirmed before being adopted. Such grouping, if incorrect, could seriously mislead plant breeders if resistance to the respective pathogens is inherited independently.

The reports of Nelson (11) and Nelson and Kernkamp (12)—that *P. trifolii* has as its imperfect stage a fungus conforming to *S. botryosum*—are not substantiated by any report in the literature. Previous workers (5, 6, 7, 8, 9) have stated that no conidial stage has ever been observed in association with *P. trifolii;* and the reports are not confirmed by the present study. Well over 400 cultures, both single-ascospore and mycelial transfers, were examined during the past 2 years, and no conidia of any description were observed. When the 2 Minnesota cultures were supplied it was stated that, though none of the cultures with which Nelson worked were available, the 2 cultures supplied were typical of those cultures. The Minnesota cultures were the same in every respect as those isolated from alfalfa in Manitoba and those obtained from Pennsylvania and Wisconsin.

Similarly, perithecia of *P. trifolii* have never been reported as the perfect stage of *S. botryosum*, whereas *P. herbarum* has been reported consistently as the perfect stage not only for the form occurring on alfalfa but for those occurring on other hosts (4, 14, 15).

At present, both the accepted system of classification and Nelson and Kernkamp's modification are being used in the literature. The 1955 list of Plant Diseases in Minnesota (13) omits *S. botryosum* and *P. herbarum* as pathogens of alfalfa, and Baxter (1) refers to the Pseudoplea stage of Stemphylium leaf spot in Iowa. Dickson's revised edition of Diseases of Field Crops (3) retains the older classification. In view of the continued need for substantiation of Nelson and Kernkamp's modification, it would appear that no change should be made in the present taxonomic position of the two organisms.

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RED CLOVER CLONES AS LOCAL-LESION HOSTS FOR BEAN YELLOW MOSAIC VIRUS[1]

Stephen Diachun and Lawrence Henson

SUMMARY

From populations of Kenland red clover, several clones that produced local necrotic lesions when inoculated with an isolate of bean yellow mosaic virus were selected. Clone KyC71-8 was useful for comparing virus infectivity in various preparations. Clones KyC40, KyC40-1, and KyC40-2 appear promising as breeding stock for resistance.

Bean yellow mosaic virus is recognized as probably the most common virus in red clover, *Trifolium pratense* L., in Kentucky (6). It is also common in red clover in some parts of the Midwest (10) and Northwest (9), and in other field legumes in the Southeast (3). Strikingly different symptoms, ranging from mild mosaic in some plants to systemic lethal necrosis in others, may develop in different plants of red clover infected with the same strain of bean yellow mosaic virus (6). Similarly, a strain of bean yellow mosaic virus can cause a wide range of symptom expressions in a genetically variable population of garden beans, *Phaseolus vulgaris* L. (1).

This paper briefly describes several clones of red clover that develop discrete local necrotic spots on leaves inoculated with bean yellow mosaic virus, indicates the usefulness of some of these clones in virus research, and suggests some of these clones as breeding material for the development of red clover varieties resistant to bean yellow mosaic virus. An abstract of some of this work has been published (5).

MATERIALS AND METHODS.—Clones of red clover were propagated by cutting vigorous young shoots from selected plants, keeping the cuttings in beakers or glasses of tap water until roots developed, and potting rooted cuttings in steamed soil. Plants were inoculated with a single-lesion isolate of bean yellow mosaic virus from a plant of red clover on the Kentucky Agricultural Experiment Station Farm, designated 204-1, that appears to be similar to strain 2 of Hutton and Peak (8) and Goodchild (7). Inoculum was prepared by crushing, with a glass spatula, young mottled leaves of infected plants of *Vicia faba* L., *Trifolium incarnatum* L., or *T. pratense* in about an equal volume of .01M sodium sulfite. Inoculum was rubbed with a glass spatula on leaves dusted with 600-mesh carborundom. Inoculated leaves were rinsed briefly with tap water 2–5 minutes after inoculation. When clones were to be compared, 3 leaves of each of 10 plants of each clone were inoculated. To minimize the deleterious effect of the aging of inoculum, the plants were distributed in replicated series or plots, each replicate inoculated with freshly prepared inoculum. Local leaf lesions were counted 6–8 days after inoculation, usually on the under surfaces, where they are more prominent than on the upper surfaces.

DESCRIPTION OF NECROTIC-SPOTTING CLONES.—Some form of mosaic developed in most of the several hundred clones of red clover that were inoculated with bean yellow mosaic virus. Local, discrete, dark, circular or angular necrotic spots (like those in Fig. 1) appeared in 4–6 days on rubbed leaves of several clones. In some plants the virus was confined in the local lesions; in others a systemic necrosis developed, resulting in the death of some plants.

Eight necrotic-spotting clones were selected for a more detailed comparison of reaction to bean yellow mosaic virus. Clones KyC2, KyC6, KyC14, and KyC71 are from plants selected from survivors of a population of seedlings of Kenland red clover inoculated with *Colletotrichum trifolii* Bain & Essary. Clone KyC71-8 is from a plant selected from a population grown from seed produced on an open-pollinated plant of clone KyC71. Clone KyC40 is from a plant selected in a field of 3-year-old Kenland in the midst of virus-infected plants. Clones KyC40-1 and KyC40-2 are from plants from seed produced on a plant of clone KyC40 growing outdoors in a cage with several other necrotic-spotting clones. These clones were selected partially because the leaf markings that occur on most plants

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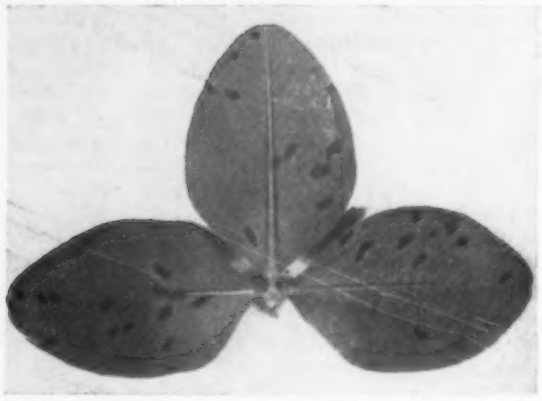

FIG. 1. Local necrotic spots on under surface of a leaf of Kenland red clover, Clone KyC71, 6 days after being rubbed with bean yellow mosaic virus 204-1.

of red clover were absent. This absence of leaf marking may serve as an aid in identifying the clones.

RESULTS.—Results of a typical inoculation test are given in Table 1. Essentially the same results were obtained in several tests, the main difference being the absolute number of local lesions, which varied markedly with different preparations of inoculum. The relative number of local lesions was essentially the same. Results of such tests under greenhouse conditions show that some of the necrotic-spotting clones, such as C71, C71-8, and C40-2, developed more local lesions than others, such as clone C6 and C14. Some clones, such as C71-8, C6, and C14, became invaded systemically sooner and in greater numbers than others, such as clones C71, C40, C40-1, and C40-2. There seems to be no positive correlation between number of local lesions and susceptibility to systemic infection: C6 produced fewer local lesions than C71 did, but more plants of C6 became systemically infected; C71-8 and C40-2 produced about the same number of local lesions, but C71-8 was consistently much more susceptible to systemic invasion.

Twenty plants of each of these 8 clones were set in the field in the spring of 1956. Natural infection with bean yellow mosaic virus occurred; symptoms were similar to those in the greenhouse. Eighteen plants died in clone KyC6, 18 in C14, 16 in C71-8, 11 in C71, 9 in C2, 3 in C40-2, 2 in C40-1, and 1 in C40. As was the case in the greenhouse, clones C40, C40-1, and C40-2 were strikingly less susceptible to systemic infection than clones C6, C14, and C71-8; C71 and C2 were intermediate, both in the greenhouse and in the field. Clone C40 and its progeny, C40-1 and C40-2, seem to have considerable promise as breeding stock for resistance to bean yellow mosaic virus. Tests with similar clones have shown that in open-pollinated F_1 progenies of clones with the necrotic reaction the number of necrotic-reacting plants was higher than in the original Kenland variety, and that polycrosses of plants with the necrotic reaction resulted in populations with an increased number of necrotic-type plants (4). Clone KyC40 and its progeny, clone KyC40-1 and KyC40-2, are not readily invaded systemically by the virus, without leaf markings, relatively vigorous, and relatively good in general appearance. Hutton and Peak have shown that the necrotic-spotting reaction to bean yellow mosaic virus in subterranean clover is controlled by a single dominant factor, and that this type of resistance is useful under field conditions (8).

TABLE 1.—*Reaction of 8 necrotic-spotting clones of Kenland red clover to bean yellow mosaic virus, isolate 204-1; 10 plants inoculated in each clone*

Clone	Local lesions per leaf	Plants with systemic necrosis	Days between inoculation and necrosis	Plants killed
KyC6	6	10	15	10
KyC14	6	10	18	10
KyC40	11	0	—	0
KyC40-1	16	0	—	0
KyC2	18	6	21	4
KyC71	22	2	39	1
KyC71-8	28	10	14	10
KyC40-2	32	0	—	0

The value of local-lesion hosts in virus research is well known. Clone C71-8 has been useful as a local lesion host for comparing the virus infectivity of different preparations of inoculum. Corbett (2) recently demonstrated that *Crotolaria spectabilis* Roth also is a local-lesion host for some strains of bean yellow mosaic virus. In our studies it was found that, in general, inoculum is more effective from young leaves than from older leaves of the same plants; inoculum is better from recently infected plants than from plants infected for some time; inoculum tends to be more active from crimson clover than from red clover; and inoculum prepared with .01M sodium sulfite tends to begin to deteriorate about 1 hour after preparation.

Necrotic-spotting clones also have proved useful as indicator plants in isolating or detecting strains of the bean yellow mosaic virus. Three distinct isolates have been collected that cause mottle (rather than necrosis) in clone KyC71-8, but necrosis in some other clones.

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EFFECT OF CERTAIN ENVIRONMENTAL CONDITIONS ON INFECTION OF WHEAT BY PUCCINIA GRAMINIS [1]

J. B. Rowell, C. R. Olien, and R. D. Wilcoxson [2]

SUMMARY

The infection process of urediospores of *Puccinia graminis* var. *tritici* race 15B on wheat seedlings (var. Little Club) was investigated. Under conditions of dew at 70°F and little or no light, urediospores germinated during the first 4 hours, a majority of the appressoria developed in the second 4 hours, and then infection processes stopped except for an occasional appressorium that formed a substomatal vesicle. Appressoria remained quiescent when the free moisture on leaf surfaces was dried slowly at 65°F under low light intensities. Such quiescent appressoria showed no detectable loss of infectivity after 3 days at 65°F in the dark under various conditions of atmospheric humidity. Rapid drying of moisture from the plant surfaces after appressoria had formed under conditions of dew resulted in a large decrease in infection. Infectivity of appressoria also diminished with prolonged dew periods exceeding 18 hours. Quiescent appressoria produced substomatal vesicles and completed the infection process after exposure to high intensities of natural light at about 85°F in the greenhouse.

A method of controlling the amount of urediospore inoculum deposited on primary leaves of wheat seedlings (8) did not assure control over the subsequent number of uredia of *Puccinia graminis* Pers. var. *tritici* (Eriks. & E. Henn.) Shear, Ball, Jacks, & Stakman in initial infection trials. Spore deposit was reproducible within a 15 per cent error with the inoculation method, and infection between replicates in single trials was reasonably uniform. However, successive inoculation trials on different days with equal numbers of freshly collected urediospores and with a uniform period of 20 hours in a constant-temperature dew chamber (70°F ± 1.5°) resulted in infection ranging from 0.1 to 10 uredia per 100 spores. Little difference was found in the per cent or rate of germination for the fresh spore collections used in the different trials. The results indicated previously unrecognized environmental effects had a major role in the infection process. Therefore, a study of the factors affecting the success of infection was undertaken. The present report describes a relationship between moisture and appressorial behavior and delineates the most favorable environmental schedule for infection based on these relationships. A preliminary account of these studies has been presented (9).

METHODS.—Primary leaves of Little Club wheat seedlings were inoculated by the procedure of Rowell and Olien (8) with 0.1 ml of a suspension of freshly collected urediospores of *P. graminis* var. *tritici* (race 15B) in a 50:50 mixture of Mobilsol 100 and U.S.P. light petrolatum oil (1 or 2 mg of spores per ml of oil). A dew chamber heated to 70° ± 1.5°F and located beneath the plant bench in a 65°F controlled-environment room was used for the moist period of the infection process. Preliminary studies had indicated that this temperature differential between the dew chamber and the room was too low (5°F) for rapid dew formation and that spore germination was low and oftentimes abnormal on surfaces introduced into the chamber dry and moistened only by dew deposition. Germination was, however, normal in amount and character when the spore-inoculated surface was moistened by an atomized spray of distilled water. Thus, inoculated plants were moistened in this manner and then placed in the dew chamber. At least 4 replicate pots were used for each environmental treatment and pots were placed at random in the dew chamber. Counts of spore germination, appressoria, and substomatal vesicles were made by microscope on strips of the lower epidermis of 5 random leaves per treatment. Uredia were counted 10–12 days after infection and were calculated as uredia per 100 spores deposited on leaves as determined by counts on leaves sampled immediately after inoculation. The data reported here are restricted to stem rust race 15B and wheat variety, Little Club, but the major effects were duplicated with another race (R111) and other host varieties (Marquis, Lee, Sentry,

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TABLE 1.—*Rate of infection of wheat seedlings from known numbers of urediospores of Puccinia graminis var. tritici with different dew periods and experimental schedules*

Dew period	Uredia/100 spores		Post-dew period at 2 relative humidities	
	Trial I	Trial II	90–100%	40–50%
Schedule I[a]				
4 hr.	5.0	4.9	3 hr.	15 hr.
6	–	2.4	1	15
8	0.8	0.6	0	15
10	–	1.2	0	13
12	1.4	1.6	0	11
Schedule II[b]				
4 hr.	0.04	0.04	0	15
5	0.1	–	0	15
6	0.2	0.2	0	15
7	0.2	–	0	15
8	0.5	0.7	0	15

[a] Plants placed in dew chamber at 9 a.m. and removed at different times.

[b] Plants placed in dew chamber at different times but removed at 5 p.m.

and Carleton), indicating that the observed effects were not limited to a single host-race combination.

One of the 2 constant-environment rooms (5) was set for a continuous temperature of 65°F and a 12-hour light period between 4 p.m. and 4 a.m., later changed to 5 p.m. and 5 a.m. The light source was daylight fluorescent tubes supplemented with 75-watt incandescent lamps. The relative humidity of the room dropped to about 50 per cent during the light period, returning to near saturation during the dark period. The dew chamber was in diffused light beneath the plant bench in this room. The second room was set for 75°F during a light period from 4 a.m. to 4 p.m., and for 65°F during the dark period. The relative humidity pattern for this room also fluctuated from about 50 per cent during the light period to near saturation during the dark.

RESULTS.—*Preliminary studies of duration of dew period.*—Incongruous results were obtained when the duration of dew period was studied (Table 1). Two schedules were followed as a means of discriminating between the effects of pre- and post-dew period environment and the actual effect of the various dew periods. For schedule I, all inoculated plants were placed in the dew chamber at the same time (9 a.m.), and the various treatments were removed at different times, while for schedule II the order was reversed: the plants were placed in the dew chamber at different times and all removed at one time (5 p.m.). The various treatments in schedule I were kept on the floor of the 65°F environment room after removal from the dew chamber and then moved to the greenhouse the following morning. In schedule II, to minimize temperature extremes, the various treatments also were stored in this environment before placing them in the dew chamber. The plants were exposed only to diffused reflected light during the lighted periods of the room. Considerably more uredia per 100 spores resulted from the 4-hour period than from longer dew periods in schedule I, but this treatment had the least infection in schedule II. The hygrothermogragh record (Fig. 1) shows that the relative humidity rapidly dropped from near saturation to 50 per cent after the light period started, at 4 p.m. All plants under schedule II were removed from the dew chamber at 5 p.m., when the room was lighted and the relative humidity was low. In schedule I the 4-hour and 6-hour treatments removed from the dew chamber at 1 p.m. and 3 p.m. were exposed, respectively, to a 3-hour and a 1-hour period of darkness and high humidity before start of the light period. Free moisture on the leaves of plants in the environment room dried in 3 hours in darkness, and in less than 1 hour during the lighted period. Therefore, the observed differences in infection were suspected to be due to these differences in the relative humidity, affecting rate of drying of the moist plant surfaces.

Effect of rate of drying on amount of infection.—Three methods were devised to test the validity of the hypothesis that dew evaporation rates affected rate of infection under the described conditions. In the first method, large-mouthed jars (Fig. 2) were used in which humidity was regulated with various solutions of sodium chloride. The jars were sealed with a cover

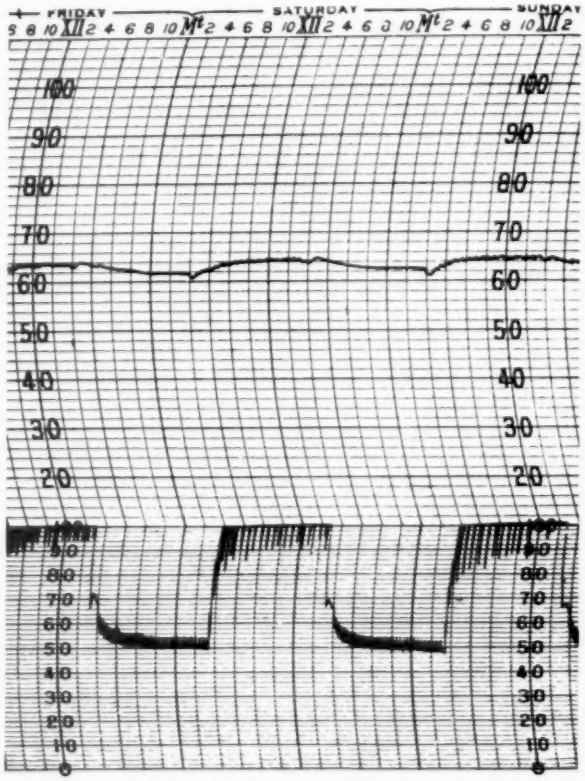


Fig. 1.—A representative hygrothermograph record for the 65°F-environment room illustrating temperature and relative humidity characteristics during the dark cycle (4 a.m. to 4 p.m.) and the light cycle (4 p.m. to 4 a.m.).

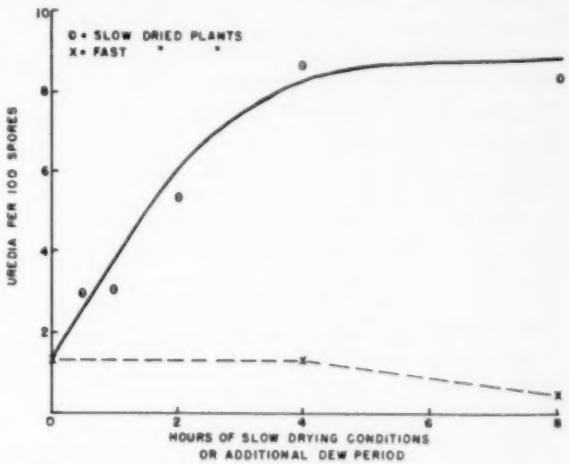
Fig. 2.—Frames for growing plants and large-mouth jars designed as controlled relative-humidity chambers used to control rate of drying of leaf surfaces. Left: holders with wheat seedlings in dish containing ½-strength Hoagland's solution. Right: position of holder with seedling leaves projecting into the jar's atmosphere during the controlled drying period.

of polyethylene film and stored 1 week in the constant-temperature room to permit atmospheric humidity in the jars to come to equilibrium. Plants were grown in special holders (Fig. 2) consisting of a plastic screen cloth frame and a double layer of filter paper. A layer of thin polyethylene film was interposed between the screen and the outer layer of filter paper to reduce evaporation losses. Germinating seeds (24 hours on moist filter paper) were inserted between the 2 layers of filter paper and the holders placed in dishes containing ½-strength Hoagland's nutrient solution. When the primary leaves were fully extended, the plants were inoculated and subjected to an 8-hour dew period, as described previously. Corks were inserted in both ends of the plant holders to prevent drying of the roots and to prevent introduction into the jars of moisture from the interior of the holders. A single holder was mounted in a hole in the polyethylene cover of each jar so that the seedling leaves projected into the atmosphere of the jar. The introduction of the moist leaves probably disrupted the humidity equilibrium of the jars, but differential rates of drying were attained. The plants were removed after 15 hours at 65°F in these chambers, and at that time the leaf surfaces in the 100 and 96 per cent r.h. series had failed to dry. These plants dried rapidly on being moved to the greenhouse. The remaining series dried at different rates, with slowest drying for the 92 per cent r.h. series, which had the greatest number of uredia (Table 2).

TABLE 2.—*Stem rust infection resulting from an 8-hour dew period followed by drying leaves at different relative humidities*

Relative humidity	Uredia/100 spores	
%	Trial I	Trial II
100	0.3	0.5
96	1.8	0.3
92	4.3	4.4
85	0.8	2.1
70	0.8	1.7

In the second method, inoculated plants were stored for various periods at 65°F in the near-saturated atmosphere of the controlled-environment room during the dark period after an 8-hour dew period and then dried in the other environment room by blowing air at 75°F and 50 per cent r.h. over the plants for 30 minutes. Dried plants were then returned to the 65°F room for the remainder of the test period. The results for a typical trial are illustrated in Fig. 3. The number of uredia per 100 spores increased directly with time up to 4 hours under slow drying conditions after removal from the dew chamber. Few uredia were produced on plants removed from the dew chamber after 8, 12, or 16



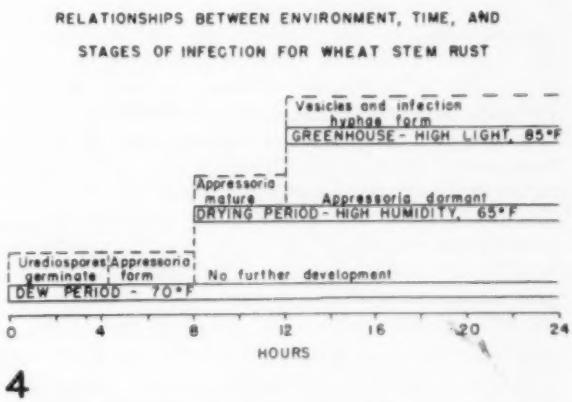


Fig. 3.—Amounts of infection developing on primary leaves of wheat seedlings inoculated with known numbers of urediospores and held for various times at slow drying conditions after an 8-hour dew period. Fast-dried plants were dried immediately after removal from the dew chamber after a dew period of 8 hours plus the indicated additional time. Fig. 4.—Schematic summary of the effects of environmental conditions on the various steps of the infection process of *Puccinia graminis* var. *tritici*.

hours and dried immediately. The ½-hour exposure to 75°F was an undesirable but unavoidable feature in these tests.

A third series of tests utilized cheesecloth layers to retard moisture loss. The inoculated plants, after an 8-hour dew period, were placed inside chambers covered with different numbers of layers of wet cheesecloth in the 65°F-environment room. The experiment was timed to complete this transfer just before start of the light period so that drying would occur while the room atmosphere was at 50 per cent r.h. Observations indicated that the approximate rate of disappearance of free moisture from the leaves increased about 1½ hours for each layer of cheesecloth. The slowest rates of drying with 2 and 3 layers of cheesecloth (Table 3) resulted in the greatest numbers of uredia. Thus, all 3 tests established a definite relation between amount of infection and rate of drying of moist plant surfaces after the described dew period.

Effect of rate of drying on development of appressoria and substomatal vesicles.—The infection process was followed by microscopic examination of the peeled epidermis of inoculated leaves using a method similar to that described by Schmitt and Staley (11). All counts were based on data from 5 random leaves. Comparison of counts made by direct observations of undisturbed leaves and of epidermal strips indicated that peeling may affect the counts of ungerminated spores but not the counts for germ tubes and appressoria. Thus, the numbers of appressoria and vesicles are expressed as percentages of germinated spores.

Observations established that under dew conditions at 70°F there is no appreciable change in the numbers of germinating spores after 4 hours of dew. Although a few distinct appressoria were present after 4 hours in the dew chamber, these structures increased in number until 8 hours (for example: after 4, 6, and 8 hours of dew period the percentage of germinated spores with appressoria was, respectively, 14, 27, and 52), after which no further increase in numbers was detected. Table 4 lists the frequencies with which appressoria and vesicles occurred at different times with fast or slow drying after the 8-hour dew period. In these experiments the dew period started at 6 a.m. and ended at 2 p.m. (0 hours in the table); the slow-dried plants remained for 3 hours in the 65°F-environment room at near-saturated r.h., while the fast-dried plants were dried by blowing air at 75°F and 50 per cent r.h. over the plants for 30 minutes, and then returned to the 65°F environment. At 5 p.m. all plants were transferred to the greenhouse (3 hours in the table). No subsequent morphological or quantitative changes were apparent for appressoria after the dew period, either with fast or slow drying. Vesicles were occasionally observed at the end of the dew period, although none of these structures was observed at that time in the experiments of Table 4. After the plants were exposed to the higher light intensities and temperatures of the greenhouse, more vesicles were found in slow-dried than fast-dried plants, indicating that drying rates affected the infectivity of the appressoria. Furthermore, the vesicles produced after fast drying apparently lacked vigor, since the difference between the numbers of vesicles formed under the different conditions was considerably less than that between the final numbers of uredia.

TABLE 3.—*Stem rust infection resulting from an 8-hour dew period followed by drying leaves in chambers covered with different layers of wet cheesecloth*

No. of layers	Uredia/100 spores	
	Trial I	Trial II
0	1.8	2.8
1	8.6	4.7
2	11.4	10.5
3	..	9.5

The results indicated that the environmental optima for appressorial formation differed from those for penetration and substomatal vesicle formation. Furthermore, appressoria, once formed, apparently remained quiescent under the dew or slow-drying environmental conditions.

Quiescence of appressoria.—The relationship between environment and appressorial behavior was investigated for prolonged periods of the dew and the slow-drying environments. Appressorial and substomatal vesicle formation was followed on epidermal strips from leaf samples in an experiment in which inoculated plants were placed in the dew chamber at 9 a.m. and a series of plants were removed after dew periods of 8, 32, and 80 hours. All plants were slow-dried overnight in the moist-cheesecloth-covered chambers and moved to the greenhouse at 8 a.m. The proportion of germ tubes with appressoria was 40, 38, and 39 per cent, respectively, at the end of dew periods of 8, 32, and 80 hours. No substomatal vesicles were observed on any of these samples or on samples collected at 8 a.m. after slow drying, and these structures appeared only after the plants had been exposed

TABLE 4.—*Observed frequencies of appressoria, substomatal vesicles, and uredia developing on wheat seedlings inoculated with wheat stem rust urediospores after an 8-hour dew period followed by fast or slow drying*

Stage	Hours after dew period	Percentages of germinated spores			
		Trial I		Trial II	
		Slow drying	Fast drying	Slow drying	Fast drying
Appressoria	0	56	51	51	49
	3	46	58	..	..
	15	57	55	53	49
	40	57	56	50	47
	67	61	58	49	50
Vesicles	0	0	0	0	0
	3	0	0	..	..
	15	6	2	0	0
	40	14	7	18	4
	67	15	7	19	6
		Per 100 spores			
Uredia	240	8.6	1.6	17.3	1.4

TABLE 5.—*Effect of extended dew periods at 70°F followed by slow and fast drying of the leaf surfaces on infection of primary leaves of wheat seedlings by stem rust urediospores*

Dew period (hours)	Uredia/100 spores	
	Slow dried	Fast dried
Series I[a]		
8	4.2	0.6
18	3.1	0.5
24	2.3	0.8
Series II[b]		
24	2.7	0.5
48	1.1	0.5
72	1.0	0.6

[a] Test plants placed in dew chamber at different times, all removed at 5 p.m.

[b] All placed in dew chamber at 5 p.m. and removed at 5 p.m. on successive days.

to a daylight period in the greenhouse. The numbers of uredia per 100 spores subsequently developing were 4.8, 1.7, and 1.5 for the respective dew periods. Thus, the data indicate that for dew periods at 70°F with little or no light the infection process stopped with the formation of the appressoria, which then became quiescent, and that dew periods exceeding 8 hours had a detrimental effect on subsequent steps in the infection process. That the infectivity of quiescent appressoria diminished with extended periods in the dew chamber was indicated by the data for a representative test (Table 5). Inoculated plants for series I were placed in the dew chamber at different times for different dew periods, but removed at the same time (5 p.m.) for constant-drying conditions overnight—in chambers covered with a double layer of wet cheesecloth for slow dried treatments, and in open chambers for fast drying. In series II of this experiment, the inoculated plants were all introduced into the dew chamber at the same time (5 p.m.), but removed at 24-hour intervals and dried as uniformly as possible in chambers in the controlled-environment room, as for series I. The numbers of uredia per 100 spores decreased with increases in length of dew period. Comparisons between the 2 series on different days appear to be valid because the amount of infection was similar for the 24-hour treatments in the 2 series. The constant but low amount of infection of fast-dried material after different durations of the dew period may have resulted from an occasional appressorium forming vesicles during the first 8 hours of the dew period.

TABLE 6.—*Infection of primary leaves of wheat seedlings by stem rust urediospores subjected to prolonged storage at 65°F after an 8-hour dew period followed by slow drying of the moist plant surfaces*

Storage period after dew period	Uredia/100 spores	Appearance of flecks
12 hours	8.1	6 days
17	5.7	7
32	6.6	7
56	6.2	8

Appressoria remained quiescent on plants maintained for prolonged periods in the environment room at 65°F following slow drying after the dew period (Table 6). These plants were inoculated and subjected to an 8-hour dew period, then removed to wet-cheesecloth-covered chambers in the environment room and allowed to dry slowly. The plants were stored for various periods on the floor of the 65°F room, where the appressoria were exposed to dark in near-saturated atmosphere alternating with low-intensity light in a 40–50 per cent relative humidity. When the plants were moved to the greenhouse the proportion of spores resulting in infection was essentially the same after the different storage periods. The long time required for the appearance of flecks indicated that the infection process was arrested during storage. In another experiment, leaves of plants were inoculated by the oil method and by brushing spores onto moistened leaves. The dew and slow-drying periods were the same as in the previous experiment. Epidermal strips examined showed that the percentage of germ tubes with appressoria after 8 and 56 hours of storage was, respectively, 60 and 40 for the oil-carried inoculum, and 63 and 53 for the brushed inoculum. A few vesicles (0.5 per cent of the germinating spores) were found at the end of the dew period, and a similar number were found on plants examined after 3 days' storage in the 65°F-environment room. After 24-hour exposure to the higher light and temperature conditions in the greenhouse, the percentage of germ tubes with vesicles increased to 6.8 and 2.5 for oil-carried inocula, and to 13 and 16 for the brushed inocula, for 8 and 56 hours of storage, respectively. Again there was a 2-day difference in flecking time for the series, and the amount of final infection was the same. Thus, appressoria on slowly dried plants remained quiescent during the storage period with low light, fluctuating relative humidity, and a temperature of 65°F, and the remaining phases of the infection process occurred under the conditions of higher light and temperature in the greenhouse.

Effect of environment on substomatal vesicle formation.—Apparently, higher temperatures and light intensities than those used in the dew chamber are required for appressoria to produce infection pegs and substomatal vesicles. In all experiments vesicles were abundant only after the plants had been exposed to daylight in the greenhouse. The importance of light for infection was also indicated by a steady decrease in the uredia produced per 100 spores for a standardized infection schedule from August through December, while the duration and intensity of natural light in the greenhouse steadily diminished (8). The maximum light intensity available in the environment rooms was only 750 foot-candles. Infection at least 10 times as great occurred in the higher light intensity in the greenhouse in repeated comparisons with that in the 2 environment rooms. Furthermore, tests in the light room with chambers having various temperature combinations ranging from 65°F to 85°F never resulted in

as much infection as occurred in the greenhouse. It is tentatively concluded that at least 1500 foot-candles of light and a temperature near 85°F are required to stimulate the formation of vesicles by the quiescent appressoria.

Discussion.—Few studies have been made of the effect of the environment on the infection process for urediospores of *P. graminis* var. *tritici*, although there is an extensive literature (2) pertaining to the post-infection effects of light, temperature, and other factors on the reaction of hosts to stem rust. Hart (1) and Hart and Forbes (3) studied the relationships between stomatal opening and closing in light and darkness and the resistance of certain varieties to infection by stem rust.

The steps of the infection process for stem rust of wheat under the conditions of the present study are summarized in Fig. 4. The steps are: 1) Urediospore germination proceeds during the first 4 hours, and appressorial formation mainly occurs between 4 and 8 hours of the dew period at 70°F. in the dark. After 8 hours, no further development in the infection process can be detected under these conditions, and the appressoria appear to be quiescent. 2) Next, slow drying at 65°F at near-saturated atmospheric humidity apparently conditions the appressoria to withstand further changes in atmospheric humidity, but no further development occurs, and the appressoria remain quiescent after the plant surfaces are dry. 3) Finally, when the plants are moved to the greenhouse and exposed to intense light and temperatures of about 85°F, the vesicles and infection hyphae form, and infection is completed.

Sharp et al (10) recently described experiments, also with urediospores of *P. graminis* var. *tritici*, that also demonstrated that the infection process under dew conditions in the dark stopped after the formation of appressoria. However, they found increased vesicle formation and subsequent infection when light and temperature were increased during the last few hours of the dew period. The results of the present study have demonstrated that the quiescent appressoria produced in an environment of dew, darkness, and temperature of 70°F lost infectivity if dried rapidly. Slow drying of plant surfaces at the end of the dew period conditioned the quiescent appressoria to survive prolonged exposure to diurnal ranges in relative humidity from 50 to near 100 per cent, low temperature (65°F), and low light intensities. The subsequent production of substomatal vesicles by the quiescent appressoria after exposure to the stronger light and higher temperatures (85°F) of a daylight period in the greenhouse apparently was an environmental response similar to that reported by Sharp et al (10) on the effect of higher light and temperatures in the last few hours of the dew period.

The oil mixture used throughout this study as a carrier for inoculating wheat seedlings with urediospores caused about half of the appressoria to form in nonstomatal areas (8). Furthermore, vesicles developed from spores dusted on leaves appear to have been more vigorous than those from spores carried in the oil mixture. However, the observed effects of different environmental conditions on the steps of the infection process were not artifacts resulting from the use of an oil carrier: with the different environmental conditions studied, the same behavior was observed on epidermal strips from plants inoculated with dry as well as oil-carried urediospores.

The efficiency of infection in these studies never exceeded 20 per cent of the deposited urediospores. Percentages of stem rust urediospores infecting the host have been reported variously as 1 (6), 4.5–7.3 (7), and 88 (4), but the environments for these tests were not described. The theoretical maximum for infection based on the number of spores that form appressoria would be about 50 uredia per 100 spores with the dew conditions studied. The nonstomatal location of 50 per cent of the appressoria for urediospores carried in the Mobilsol 100 and U.S.P. light petrolatum oil mixture would be expected to reduce the infection rate by one half. Actually, the most frequent rates of infection in these studies were 5–10 uredia per 100 spores. It is not known whether the observed low efficiency of infection resulted from the oil carrier, suboptimum environmental conditions, or both.

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THE ROLE OF PECTIC AND CELLULOLYTIC ENZYMES IN PATHOGENESIS BY PSEUDOMONAS SOLANACEARUM [1]

Akhtar Husain and Arthur Kelman [2]

SUMMARY

Liquid culture filtrates of 3 strains of *Pseudomonas solanacearum* differing in pathogenicity contained pectinmethylesterase (PME), polygalacturonase (PG), and a cellulolytic enzyme (Cx). Stem tissues of tomato cuttings placed in these culture filtrates were rapidly macerated. When 6-week-old cuttings were placed in culture filtrates of weakly pathogenic or nonpathogenic strains containing these 3 enzymes, wilting symptoms did not appear within 24 hours. Small succulent cuttings wilted slightly following extensive maceration of stem tissues immersed in culture filtrates. Both heated and unheated solutions of a commercial pectic enzyme preparation (Pectinol 100D) caused wilting and necrosis of leaflets. Unheated solutions caused greater injury than did the heated solutions, and also caused maceration of the basal portions of the cuttings. In a highly purified preparation of fungal PG, no wilting occurred although stems of cuttings were macerated after 48 hours. At low concentrations, a highly purified preparation of cellulase from *Myrothecium verrucaria* caused leaves of young tomato cuttings to wilt in 12 hours. Microchemical tests and microscopic observations on tissues from tomato and tobacco plants infected by *P. solanacearum* revealed that both cellulosic and pectic materials in the host tissues were affected, indicating that specific enzymes for these substrates are active *in vivo*. A similar breakdown of pectic and cellulosic materials was observed in cuttings treated with culture filtrates of *P. solanacearum* and solutions of purified enzymes from other sources. Plugs that stained with ruthenium red were observed in cuttings treated with heated as well as with unheated culture filtrates or with solutions of purified PG. These results indicate that the main role of the pectic and cellulolytic enzymes of *P. solanacearum* in pathogenesis involves the breakdown of host tissue.

In plants affected by the causal agent of Granville wilt, *Pseudomonas solanacearum* E. F. Sm., breakdown of parenchyma tissue in the vascular bundles and adjacent tissues of stems and root is commonly observed (15). In young and succulent plants, the decay of pith and cortical tissue near the vascular bundles is more pronounced than in older plants. This decay is associated with the complete collapse and rapid death of diseased plants.

Various suggestions have been made to explain the mechanism of tissue breakdown caused by this bacterium, but none of these has been substantiated by experimental data. Hutchinson (13) isolated 5 species of saprophytic bacteria from infected tobacco plants and suggested that the activity of these secondary invaders caused the rotting of tissues already invaded by *P. solanacearum*. On the basis of histological examinations, Hutchinson considered that enzymes produced by the pathogen also may be important in causing the dissolution of middle lamellae. Smith (29) observed and described in detail the extensive breakdown of vascular bundles in various plants affected by *P. solanacearum*. He also noted that middle lamellae were dissolved and suggested that the development of dense masses of bacteria in the vessels resulted in rupture of cells.

In a study of several vascular parasites and certain other plant pathogens, Winstead and Walker (32) found that *P. solanacearum* produced PME (pectinmethylesterase) and PG (polygalacturonase) in culture. They postulated that these enzymes may play a role in the rapid spread of bacteria from cell to cell and that enzymatic activity also may be related to the rapid wilting of plants infected by this organism.

In other diseases of plants where rotting of tissue is associated with invasion by soft-rot bacteria, pectic enzymes have been regarded as the main causes of tissue breakdown (33, 34).

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Although the production of pectic enzymes and a cellulolytic enzyme (Cx) by *P. solanacearum* has been demonstrated in culture (10) and in diseased plants (11), the properties of these enzymes and their roles in pathogenesis by this organism have not been studied extensively. The main objectives of the present investigation were to make a detailed study of the production of pectic and cellulolytic enzymes by *P. solanacearum* and to determine certain of their functions in the disease syndrome.

MATERIALS AND METHODS.—A pathogenic (F-1), a weakly pathogenic (B-2), and a nonpathogenic (B-1) strain of *P. solanacearum* were used in these studies. The characteristics of the different strains, cultural procedures, and wilt-inducing effects of culture filtrates on tomato and tobacco cuttings were described in a previous paper (12).

In the preparation of culture media for enzyme assays, 10 ml of a bacterial suspension was added to 1-liter flasks containing 400 ml of a casein-hydrolyzate yeast-extract mineral nutrient medium (12). After 24 hours on a rotary shaker at 32°C, the culture medium was centrifuged at 5000 rpm for 1 hour and the clear supernate was used in tests for enzyme activity. When stored, the sample was covered with a layer of toluol and held at 5°C.

Pectinmethylesterase (PME) activity was determined by estimating mg of methoxyl groups per ml of culture filtrate released in 30 minutes from a 1 per cent solution of citrus pectin (California Fruit Growers Exchange) following the continuous titration method of Kertesz (16).

Polygalacturonase (PG) was assayed by estimating the loss in viscosity of the substrate and determining the reducing groups formed. The loss in viscosity was estimated in size-300 Fenske-Ostwald viscometers in a water bath at 30±0.1°C (4). Solutions (1.2 per cent) of sodium polypectate and of citrus pectin adjusted to pH 5.5 and containing citrate buffer were used as substrates. Reducing groups were determined by the Willstätter-Schudel hypoiodite method as modified by Jansen and MacDonnell (14).

The hydrolytic products of sodium polypectate were analyzed by 1-dimensional descending paper chromatography. Twenty ml of the culture medium containing the enzyme was added to 80 ml of a 1 per cent solution of sodium polypectate adjusted to pH 5.5. The reaction mixture was incubated under aseptic conditions at 30°C in a water bath. Five-ml samples were removed at 12-, 24-, and 48-hour intervals; enzymatic activity was stopped by boiling for 10 minutes, and the samples were stored at 5°C before use.

In the preparation of chromatograms, a small quantity of each sample was applied with a capillary pipette to Whatman No. 1 paper strips with a 1 per cent solution of D-galacturonic acid as a standard. Hydrolyzates obtained from the action of a purified fungal PG on the substrate and a heat-inactivated enzyme-substrate mixture were used as controls. A 4:1:5 mixture of 1-butanol, acetic acid, and water was used as the solvent (20), and the chromatograms were allowed to run for 24–36 hours at 25°C. The dried chromatograms were developed by treating with benzidine (2) or aniline phthalate (21), and heating the paper strips at 105°C for 5–10 minutes.

Activity of the cellulolytic enzyme (Cx) was determined by the cup-plate method of Dingle, Reed, and Solomons (7), and by the measurement of loss in viscosity of the substrate (18). Viscosity was measured in a manner similar to that described for PG assay with the exception that a 1.2 per cent solution of carboxymethylcellulose (CMC 70-Premium, Medium Viscosity, Hercules Powder Co.) at pH 5.5 with a citrate buffer was used as the substrate (4).

A chromatographic analysis was also made of the hydrolytic products resulting from the action of the Cx enzyme of *P. solanacearum* on CMC. Twenty ml of the culture filtrate was added to 30 ml of a 1 per cent solution of CMC adjusted to pH 6.5. The reaction mixture was incubated under aseptic conditions at 30°C (±1.0). Five-ml aliquots were removed at 24-, 48-, and 72-hour intervals, boiled to inactivate the enzyme, and stored at 5°C. Different samples were applied on Whatman No. 1 paper strips in the manner described above. One per cent solutions of glucose and of cellobiose were used as standards. In addition, hydrolyzates obtained 1) from a commercial cellulase-substrate mixture and 2) from the heat-inactivated enzyme-substrate mixture also were applied to the paper strip for comparison. One-dimensional descending chromatograms were run at 25°C for 16–24 hours; a mixture of 2-propanol and acetic acid was used as the solvent (17). Chromatograms were developed in the manner described above for chromatograms of PG.

To determine the role of enzymes in the disease syndrome, tomato cuttings were placed in different solutions sterilized by filtration and containing enzymes from *P. solanacearum*, enzymes obtained from commercial sources, or purified enzymes from other investigators. Inasmuch as a heat-stable material that caused rapid wilting of tomato cuttings was present in the culture filtrate of the highly pathogenic F-1 strain (12), culture filtrates of the nonpathogenic B-1 strain and of the weakly pathogenic B-2 strain were used mainly in studies on wilt induction. Previous work on the mechanism of wilting had shown that the culture filtrates of B-1 and B-2 were free of the heat-stable wilt-inducing material (12), although PME, PG, and the Cx enzyme were present.

Seedlings of 2 different sizes and ages were used as test plants. One group of plants consisted of young, succulent seedlings, 6 in. tall and 4–5 weeks old. The second group was older, less succulent plants, 10 in. tall and 6–8 weeks old. Bases of cuttings were immersed for 24–48 hours in the different sterile solutions, with solution replacement every 12 hours. In addition to culture filtrates of *P. solanacearum*, a 0.5 per cent solution of Pectinol 100D (Rohm and Haas Co.) and 0.005–0.05 per cent solutions of a highly purified fungal PG obtained from Dr. E. F. Jansen also were

used. All solutions were first filtered through filter paper and then passed through Seitz filters. A portion of each solution was autoclaved at 18 lb. pressure for 20 minutes. The effects of these enzyme solutions on tomato cuttings were determined in the same manner as that used for the culture filtrates.

The effects of a highly purified cellulase of *Myrothecium verrucaria* (Alb. & Schw.) Ditm. ex Fr. obtained from Dr. D. R. Whitaker were also evaluated. Inasmuch as a limited quantity of this cellulolytic enzyme was available, only cuttings from young seedlings were used, and solutions containing concentrations of 0.005–0.01 per cent were tested. A numerical rating system was used to evaluate effects of the different enzyme preparations on all cuttings (12).

Samples for histological studies were taken from the following plant materials: 1) stem sections from the apical portions of diseased tomato and tobacco plants removed 1 week after inoculation with the F-1 strain of *P. solanacearum;* 2) similar stem pieces from healthy tomato and tobacco plants; 3) tomato cuttings treated with heated and unheated culture filtrates for 24 hours; and 4) tomato cuttings treated with heated and unheated solutions of purified PG for 48 hours. All stem sections were cut into 1-cm segments, fixed in a formalin, acetic acid, alcohol solution for 48 hours, dehydrated in a 1-butanol series, and embedded in paraffin. Transverse sections 10–12 μ thick were cut with a rotary microtome.

To determine the effect of enzymes on pectic substances in plant tissues, the sections were stained with a 1:5000 aqueous solution of ruthenium red (23), washed in water, passed through ethanol-xylene series, and mounted in Permount. Freehand sections of fresh material also were stained with ruthenium red and examined to determine whether the pectic substances of the cell walls or middle lamellae had been affected.

To determine whether the cellulosic components of cell walls were also affected, freehand and microtome sections of diseased tobacco and tomato plants, as well as sections from cuttings treated with different enzyme solutions, were examined under the polarizing microscope. Loss of birefringence in cell walls was interpreted as evidence of the degradation of cellulose (23).

RESULTS.—Culture filtrates of the highly pathogenic F-1 strain contained significant amounts of PME. Enzyme activity of the culture medium was higher than that of a 1 per cent solution of commercial PME (Pectinesterase 5, Rohm and Haas Co.). The weakly pathogenic B-2 strain produced considerably less PME than did the F-1 strain (Table 1).

Results of viscosity measurements and reducing group determinations showed that both strains produce PG. The filtrates from both strains caused a loss in viscosity of solutions of citrus pectin and sodium polypectate. However, the rate of loss in viscosity was considerably slower with citrus pectin as a substrate than with sodium polypectate (Fig. 1,A). Reducing groups could be detected after 1 hour of incubation and increased rapidly during a 5-hour period (Fig. 1,B). The levels of reducing groups continued to increase, but less rapidly, during the next 7 hours. The weakly pathogenic B-2 strain consistently produced higher levels of PG than did the more pathogenic F-1 strain (Table 1).

TABLE 1.—*Comparative production of pectic (PME and PG) and cellulolytic (Cx) enzymes by a highly pathogenic F-1 and a weakly pathogenic B-2 strain of Pseudomonas solanacearum*

Enzyme source	Relative growth[a]	Relative enzyme activity[b] PME[c]	PG[d]	Cx[e]
F-1 culture filtrate	45	31	50	222
B-2 culture filtrate	46	8	166	17

[a] Relative growth in terms of turbidity of 1:10 dilution of culture liquid as measured on logarithmic scale of Fisher electrophotometer with the 425-mμ filter.
[b] Results represent means of 3 experiments.
[c] Mg of methoxyl groups formed per ml of culture filtrate in 30 minutes $\times$ 10.
[d] Reciprocal of time for 50 per cent loss in viscosity of 1.2 per cent sodium polypectate $\times$ 100.
[e] Reciprocal of time for 50 per cent loss in viscosity of 1.2 per cent carboxymethylcellulose $\times$ 1000.

In addition to the 2 pectolytic enzymes, culture filtrates of the 2 strains contained the cellulolytic enzyme (Cx) that is capable of causing a rapid loss in viscosity of CMC solutions (26). However, in all tests, assays revealed much higher levels of the Cx enzyme in culture filtrates of the F-1 strain than in filtrates of the less pathogenic strain (Table 1). The culture filtrate of the F-1 strain contained levels of the Cx enzyme adequate to cause a 60 per cent reduction in viscosity of a 1.2 per cent solution of CMC within 5 minutes (Fig. 1,C).

Presence of the Cx enzyme in culture filtrates was further confirmed by the cup-plate method. The culture filtrate of the F-1 strain produced a zone of 12.5 mm on cup plates. This was comparable in activity to a 1 per cent solution of commercial cellulase (Enzyme 19-AP, Rohm and Haas Co.) that produced a zone 18.4 mm in diameter.

Additional tests with the nonpathogenic B-1 strain, following the above procedures, indicated that this strain also produced PME, PG, and the Cx enzyme.

Properties of PME from the virulent F-1 strain.—The influence of pH on enzyme activity of the filtrate was studied by adjusting the substrate to different pH values with 0.1N HCl or 0.1N NaOH and following the procedure outlined previously for PME determinations. Relative enzyme activity was estimated as mg of methoxyl groups released per ml of culture filtrate in 30 minutes $\times$ 100. The PME produced by *P. solanacearum* has a wide pH range, showing significant activity from pH 4.5 to pH 8.0, with an optimum between pH 6.5 and pH 7.0 (Fig. 1,D).

In studies on the effects of inorganic salts on enzyme activity, the filtrate was dialyzed against distilled water at 5°C for 24 hours; the water was changed at 12-hour intervals. Salts were added to the substrate in differ-

ent concentrations, and enzyme activity was remeasured as described previously. Both sodium chloride and potassium chloride activated the enzyme (Table 2).

Properties of PG from the virulent F-1 strain.—Chromatographic analysis of hydrolytic products of the sodium polypectate solution revealed that galacturonic acid was produced after 12 hours of incubation of the substrate with culture filtrate from *P. solanacearum*. Galacturonic acid levels increased with time, reaching the greatest concentration at 48 hours. In addition to galacturonic acid, an intermediate product of hydrolysis with an R_f value of 0.03 was also detected on the chromatogram. In chromatograms obtained with the highly purified fungal PG prepared by Dr. Jansen, galacturonic acid alone was present after 48 hours of incubation, and intermediate products of hydrolysis were not detected.

In experiments to determine the pH optimum of PG in the culture filtrate, solutions of sodium polypectate were adjusted to different pH values. McIlvain's citrate buffer (19) was used in the range of pH 3.0–7.0, and Sorenson's borate buffer (5) above pH 7.0. Enzyme activity was determined by viscosity measurements. Fifty per cent loss in viscosity was

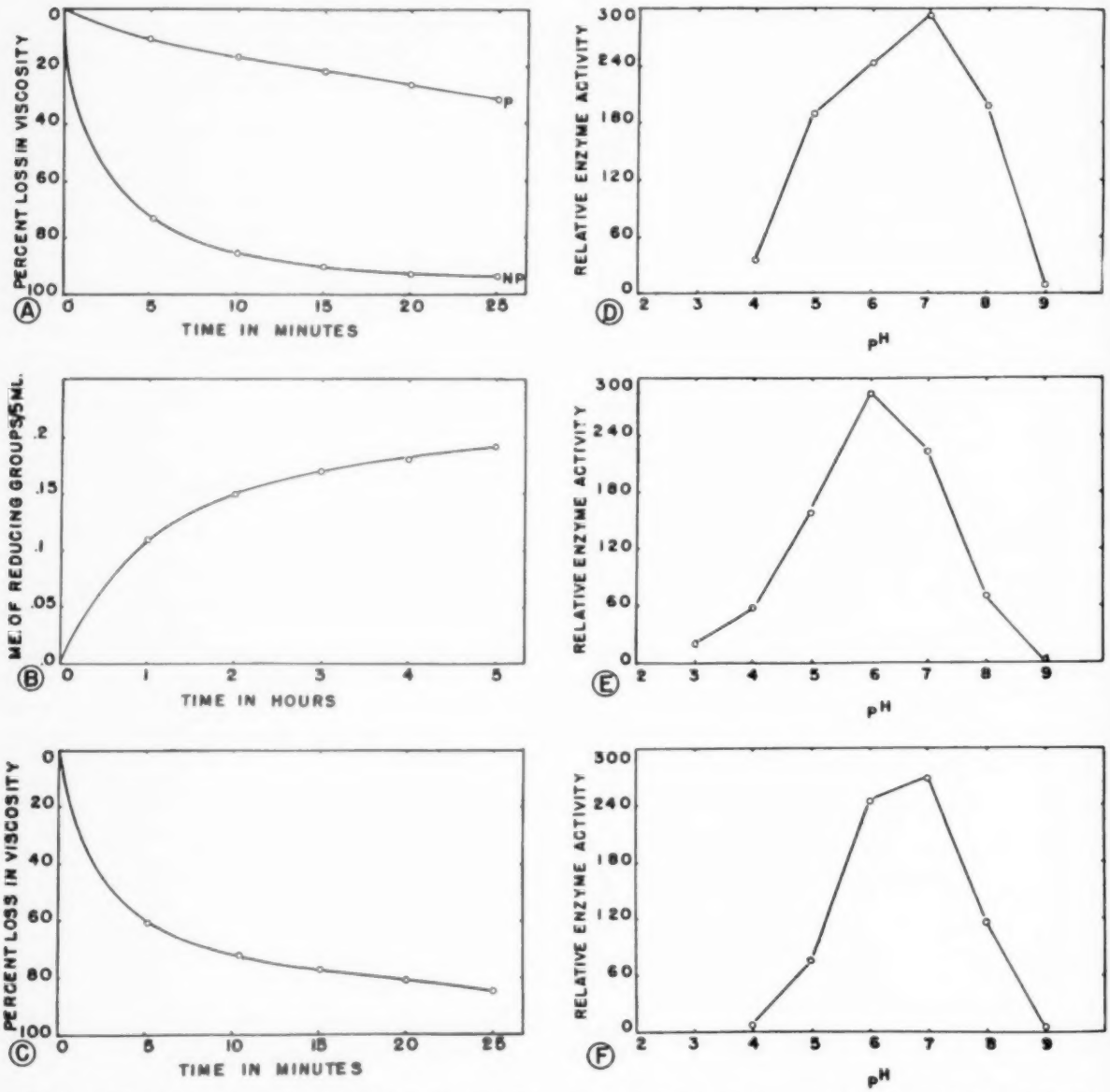

FIG. 1. Effects of culture filtrates of the virulent F-1 strain of *Pseudomonas solanacearum* on A) loss in viscosity of 1.2 per cent pectin (P) and sodium polypectate (NP); B) release of reducing groups from 0.5 per cent solution of sodium polypectate; and C) loss in viscosity of 1.2 per cent solution of carboxymethylcellulose. Influence of pH on activity of D) pectinmethylesterase (PME); E) polygalacturonase (PG); and F) of a cellulolytic enzyme (Cx) in culture filtrates of the F-1 strain of *P. solanacearum*.

TABLE 2.—*Relative pectinmethylesterase activity of culture filtrates of Pseudomonas solanacearum in presence of different salts with citrus pectin as a substrate*

Concentration of salt added	Relative enzyme activity[a]
No salt	25
0.2 M NaCl	34
0.4 M NaCl	49
0.2 M KCl	47
0.4 M KCl	50

[a] Mg of methoxyl groups formed per ml of culture filtrate in 30 minutes × 10.

taken as an arbitrary unit, and relative enzyme activity was expressed as the reciprocal of time taken for 50 per cent loss in viscosity × 100. Results show that the enzyme is active between pH 3.0 and pH 9.0, with optimum near pH 6.0 (Fig. 1,E).

In studies on the effect of inorganic salts on enzyme activity, the culture filtrate was dialyzed against a large volume of distilled water for 48 hours. Different salts were added to the substrate, and enzyme activity was determined by viscosity measurements. Relative enzyme activity was estimated in the manner described above. Enzyme activity was decreased by dialysis, and it was not restored by addition of sodium or potassium chloride.

Properties of Cx from the virulent F-1 strain.—Examination by paper chromatography of the products of enzymatic hydrolysis of CMC by culture filtrates revealed that only cellobiose was produced after a 72-hour incubation period. On the chromatogram, some reducing compounds of presumably large molecular size that did not move from the point of application of the sample were detected.

The pH optimum of the Cx enzyme was determined in a manner similar to that described for PG; a 1.2 per cent solution of CMC was used as the substrate. The Cx enzyme was active between pH 4.0 and pH 9.0, with optimum at pH 7.0 (Fig. 1,F).

Effects of different metal salts on Cx enzyme activity of culture filtrates were also determined in a manner similar to that described for PG. Slight activation was observed with sodium chloride and potassium chloride, but salts containing heavy metals such as copper, mercury, and zinc inhibited enzyme activity at very low concentrations (Table 3).

Effects of culture filtrates and other enzyme solutions on tomato cuttings.—Cuttings from 10-in. and 6- to 8-week-old tomato plants did not wilt when placed in culture filtrates of either strain B-1 or B-2 for 24 hours. Cortical tissues of lower portions of these cuttings were macerated during a 12-hour period, indicating activity of pectolytic enzymes in the culture filtrates. After 24 hours, the entire basal portions of cuttings immersed in the solutions were very soft or partially decomposed. Often portions of the epidermal and cortical tissue were sloughed off. Transverse sections of stems showed light-brown water-soaked areas in the vascular region. At these points, the vascular tissue was broken down. Slight curling and necrosis of leaf margins and tips were also evident after 24 hours. At this time, marked leaf epinasty was evident. Occasionally, internal tissue collapsed in the terminal portions of cuttings, and the stems broke at this point. Although severe epinasty of leaves became evident in all cuttings placed in the heated culture filtrates, no maceration of tissues or other injury was observed.

With cuttings from seedlings (6 in. tall and 4- to 5-weeks old), the lower portions were completely macerated. Often all the cortical and epidermal tissues immersed in the solution were sloughed off and the terminal buds and leaf petioles collapsed. Cuttings showing this extensive damage to stem tissue were partially wilted (mean wilt index for 5 cuttings = 2.5) after 18–24 hours. No symptoms of a similar nature were observed in cuttings placed in heated culture filtrates.

Maceration of stems of cuttings placed in culture filtrates of the virulent F-1 strain was also very rapid. As was noted previously (12), cuttings wilted in heated and unheated culture filtrates of the virulent strain, although rate of development of symptoms in the heated filtrate was slower.

Cuttings (obtained from both large and small tomato plants) placed in heated and unheated solutions of Pectinol 100D showed severe necrosis and wilting of leaflets within 24 hours. After 48 hours, the cuttings placed in unheated solutions showed some drooping of petioles, and the basal portions of the stems immersed in the solution were macerated; these symptoms were not seen in cuttings in heated solutions. The amount of maceration of cuttings in the Pectinol 100D solution was much less than that observed in cuttings placed in culture filtrates of *P. solanacearum*, although PG activity of the commercial enzyme sample was 20 times as great as that of the culture filtrate of the weakly pathogenic B-2 and nonpathogenic B-1 strains of the bacterium. After 48 hours, the mean wilting index of the cuttings in the unheated Pectinol 100D solution was 4.5, and that of cuttings in the heated was 3.5. Apparently, non-enzymatic components in the Pectinol solution were involved in this wilting.

Cuttings treated with the purified fungal PG showed no evidence of wilting after 48 hours. Slight necrosis of margins and tips of leaves was observed in both

TABLE 3.—*Relative cellulase (Cx) activity of culture filtrates of Pseudomonas solanacearum in the presence of various salts with carboxymethylcellulose (CMC) as a substrate*

Reaction mixture	Relative enzyme activity[a]
Nondialyzed filtrate + CMC	45
Dialyzed filtrate + CMC	40
Dialyzed filtrate + CMC + 0.1M NaCl	50
Dialyzed filtrate + CMC + 0.1M KCl	55
Dialyzed filtrate + CMC + 0.02M $HgCl_2$	2
Dialyzed filtrate + CMC + 0.02M $CuSO_4$	0
Dialyzed filtrate + CMC + 0.02M $ZnCl_2$	5

[a] Reciprocal of time for 50 per cent loss in viscosity of a 1.2 per cent solution of CMC × 100.

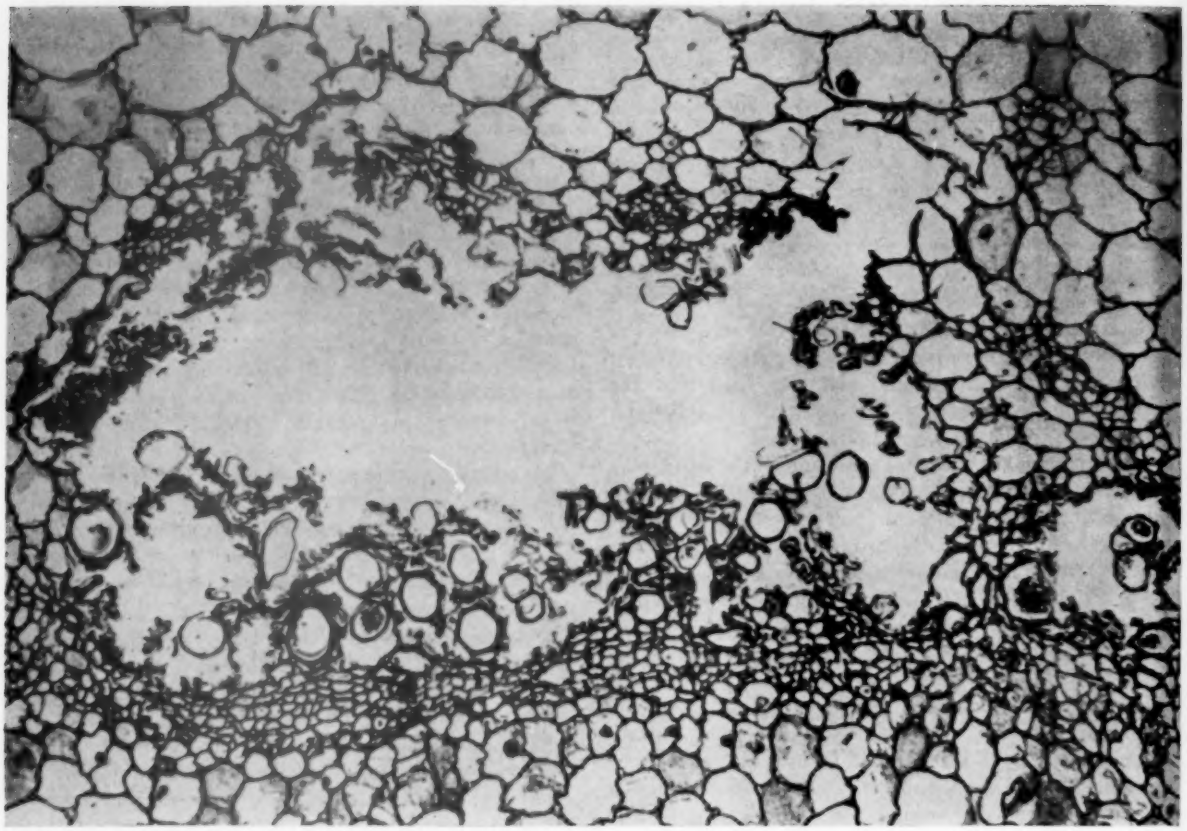

FIG. 2. Transverse section of a diseased tomato stem (infected by *Pseudomonas solanacearum*) stained with ruthenium red, showing disorganization of vascular tissue caused by breakdown of pectic and cellulosic constituents of cell walls.

heated and unheated solutions. Maceration of the vascular tissues of the stem occurred in cuttings placed in unheated solutions after 18 hours, although only slight decomposition of the bases of cuttings was evident. This maceration was less than that caused by culture filtrates of the different strains of the wilt bacterium or by Pectinol 100D. Vascular browning of cuttings developed both in heated and unheated solutions of the purified PG.

Purified Myrothecium cellulase caused small tomato cuttings to wilt within 4 hours. First symptoms were noticed in the youngest leaves; these were followed by necrosis of margins and tips of lower leaflets. Only slight drooping of petioles was observed after 10 hours. The wilt index after 12 hours was 3.5 (mean of 5 cuttings). No injury was noticed in cuttings placed in heat-inactivated samples even after 24 hours.

All of the above experiments on the effect of culture filtrates and other enzyme solutions on cuttings were repeated at least 3 times, and similar results were obtained in each trial.

Histological studies.—Considerable breakdown of pectic material in the middle lamellae of parenchyma cells adjacent to xylem vessels of infected tomato and tobacco plants was evident in fixed paraffin or fresh freehand sections stained with ruthenium red. Similar effects on pectic substances were also evident in phloem parenchyma and pith. Occasionally, cortical tissue also was disorganized. In advanced stages of invasion, all elements of the vascular bundles were broken down and disorganized, with the resultant formation of large cavities (Fig. 2). The pectic substances of the middle lamellae of cells were mainly affected, and pectic compounds in cell walls were affected only in the centers of cavities. Gums or deposits containing pectic substances were found only occasionally in the vessels observed in fresh freehand sections. In paraffin sections, however, a large number of xylem vessels were filled with a granular material that was readily stained with ruthenium red. Large numbers of bacterial cells were present in the vessels, and these bacteria stained readily with ruthenium red (Fig. 3,A).

A similar breakdown of pectic substances was also noted in sections obtained from tomato cuttings treated with a culture filtrate of *P. solanacearum* (Fig. 3,C) and also with a solution of the purified PG (Fig. 3,E). Breakdown of tissue was much more pronounced in cuttings treated with culture filtrates of *P. solanacearum* than in those treated with the purified fungal PG solution. No breakdown of tissue was observed in cuttings placed in either the heated culture filtrate (Fig.

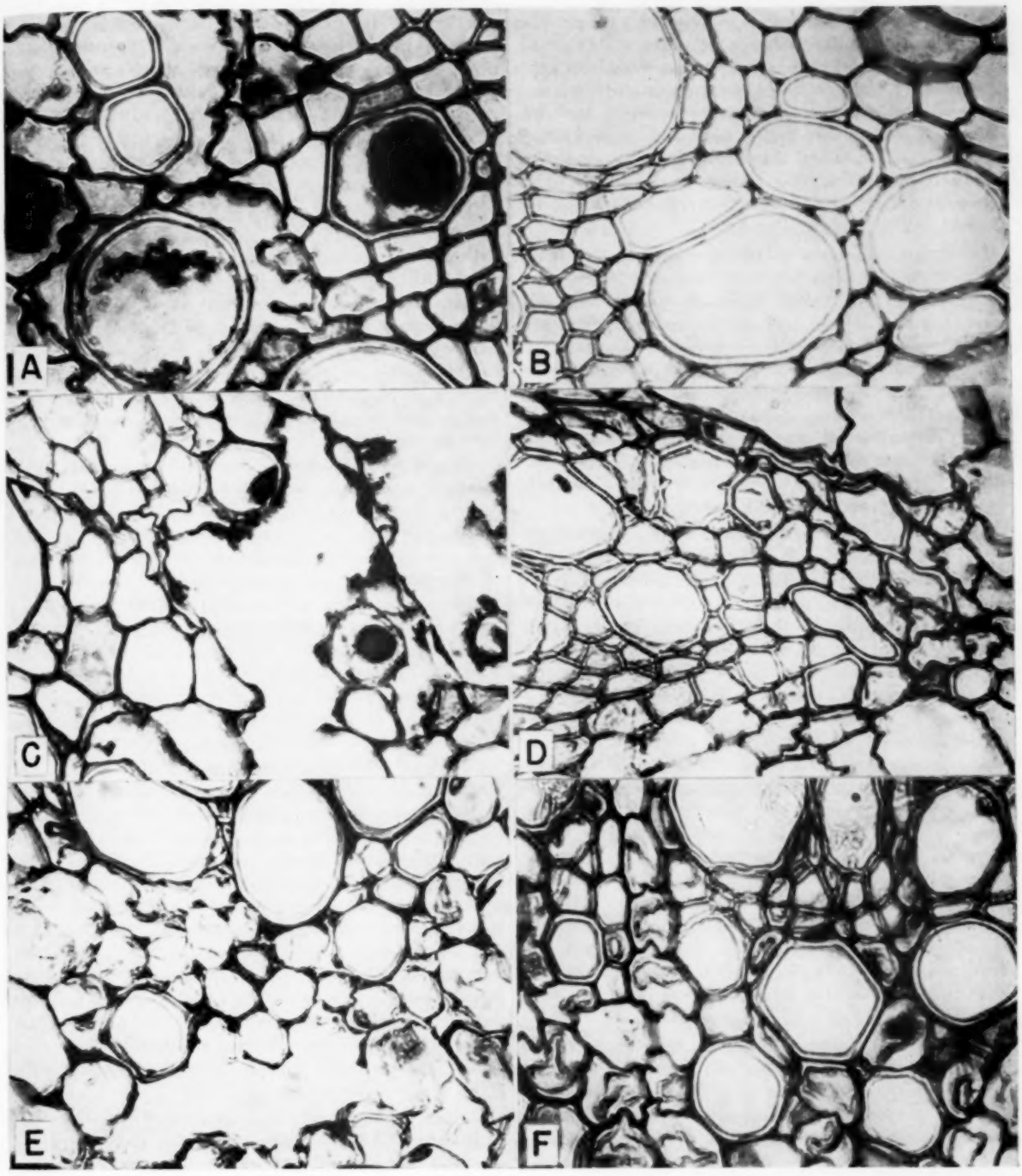

FIG. 3. Photomicrographs of transverse sections of tomato stem tissue. A) A plant infected by *Pseudomonas solanacearum* (showing dissolution of pectic substances in the middle lamellae and separation of parenchyma cells from xylem vessels of a vascular bundle). B) A healthy plant (showing intact parenchyma cells and xylem vessels in a vascular bundle). C-F) Cuttings of tomato treated with: C) an unheated culture filtrate of B-2 strain of *P. solanacearum* (showing breakdown of pectic substances in the middle lamellae and cell walls and the separation of cells in a vascular bundle); D) a heated culture filtrate (showing intact cells of a vascular bundle); E) an unheated solution of a purified PG (showing breakdown of pectic substances of the middle lamella and cell walls and the separation of cells in a vascular bundle); and F) a heated solution of purified PG (showing intact cells of a vascular bundle).

3,D) or in a heat-inactivated solution of purified PG (Fig. 3,F). Gum-like deposits or plugs that stained with ruthenium red were seen in xylem vessels of cuttings treated with unheated culture filtrates and enzyme solutions; however, these same types of deposits were also formed in cuttings treated with heated solutions, an indication that these deposits were not the direct result of enzyme action. In every case, only a small number of vessels were plugged, and a majority of them were free from any deposit.

Examination under a polarizing microscope of both freehand and paraffin sections from infected tomato plants showed that xylem vessels as well as adjacent parenchyma cells had lost the typical birefringence characteristic of cell walls in healthy tissues. This indicates that cellulosic constituents of cell walls were affected (23). Digestion of cellulose was noticed only in those areas of the stem where a cavity formed. Similar loss in birefringence of cell walls was also seen in sections obtained from tomato cuttings treated with culture filtrates of *P. solanacearum* or with a solution of purified Myrothecium cellulase.

Discussion.—From these studies on the pectic and cellulolytic enzymes of *P. solanacearum* certain conclusions can be made relative to the mechanisms involved in 2 major phases of pathogenesis: induction of wilting and decomposition of tissues. The data obtained in this investigation, as well as evidence as to the role of bacterial slime in wilting (12), indicate that pectic enzymes of the wilt bacterium were not involved in the wilting process in the manner postulated by Gothoskar et al (8) for the pectic enzymes of *Fusarium oxysporum* Schlecht. f. *lycopersici* Sacc. These authors suggested that wilting results from a gradual occlusion of xylem elements following the release of large fragments of pectic materials from cell walls by the action of pectolytic enzymes. It also has been suggested that pectic enzymes play a similar role in the wilting caused by other species of *Fusarium* (32), by *P. solanacearum* (32), and by *Verticillium albo-atrum* Reinke & Berth. (27). Further support for this concept in relation to Fusarium wilt of tomato has been presented by Pierson, Gothoskar, and Walker (22), on the basis of a histological study.

Although Waggoner and Dimond (30) demonstrated that pectic enzymes were formed by the Fusarium wilt fungus in culture and that PME and possibly PG were present in diseased plants, these authors pointed out that the specific role of pectic enzymes in the mechanism of wilting by *F. oxysporum* f. *lycopersici* had not been conclusively determined.

The conclusion that pectic enzymes do not play a major role in wilting of plants invaded by *P. solanacearum* is based on the following observations: Culture filtrates of non-slime-forming weakly pathogenic or nonpathogenic strains contained pectic enzymes but did not induce wilting symptoms in large tomato cuttings. When very young and succulent tomato cuttings were placed in a culture filtrate of the weakly pathogenic strain that contained high levels of PG, they wilted slightly only after very extensive maceration of stem tissues had occurred. Inactivation of pectic enzymes by heating a culture filtrate of the highly pathogenic F-1 strain resulted in only a slight reduction in the wilt-inducing properties of this filtrate (12). Furthermore, a solution of a highly purified fungal PG did not cause wilting, even though observations of sections taken from treated cuttings showed that pectic materials in the vascular tissues were decomposed.

Deposits of pectic substances sufficient to cause wilting were not observed in the vessels of 1) infected plants, 2) cuttings treated with culture filtrates, or 3) those treated with a highly purified fungal PG. Deposits that stained with ruthenium red were formed in the vessels of plants treated with either culture filtrates or purified PG; however, such deposits were also formed in cuttings placed in solutions that had been treated to inactivate the enzymes. Previously, Waggoner and Dimond (30) also had observed that solutions containing heat-inactivated pectic enzymes caused the formation of vascular deposits or plugs in tomato cuttings. These observations of the formation of deposits with heated samples differ from those of Pierson, Gothoskar, and Walker (22). In their studies on Fusarium wilt of tomato these workers observed that such deposits were not found when enzymes in culture filtrates or commercial samples were inactivated by heating before use. Since the specificity of ruthenium red for pectic substances may not be reliable in all instances (16), it is also possible that deposits observed in fixed sections stained with this dye may not be pectic in nature.

Of the various enzymes studied, only the purified Myrothecium cellulase caused wilting of small tomato cuttings when tests were made using very low concentrations of this enzyme. Although evidence was obtained that the cellulolytic enzyme formed by *P. solanacearum* in culture differed in certain characteristics from the cellulase of *M. verrucaria*, the possibility exists that the cellulolytic enzymes of *P. solanacearum* may play a subsidiary role in the wilting mechanism. In this connection it is of interest to note that a slight decrease in wilt-inducing effect followed the heating of culture filtrates of the highly pathogenic F-1 strain. Before heating, these culture filtrates contained high levels of the Cx enzyme.

In the chromatographic analysis of products of hydrolysis of CMC by the Cx enzyme of *P. solanacearum*, it was found that cellobiose was formed. However, no glucose was detected. It has not been shown as yet that the Granville wilt bacterium can utilize cellulose or cellobiose as carbon sources (15). Thus, it is difficult to visualize the role of cellulolytic enzymes in the nutrition of this bacterium.

In general the Cx enzyme is formed by most cellulolytic organisms only in the presence of cellulose or cellulose derivatives (25). In these studies relatively high levels of the Cx enzyme were formed in a medium that contained no cellulose or derivatives of cellulose.

The optimum pH of the Cx enzyme of the wilt bacterium (about pH 7.0) is above that reported for the activity of cellulolytic enzymes produced by most microorganisms (26, 28). Activity of the Cx enzyme was reduced greatly in the presence of salts of heavy metals, however. Basu and Whitaker (3) obtained similar results for the cellulase of *M. verrucaria*.

It is well known that many non-cellulolytic organisms produce enzymes that are capable of hydrolyzing soluble cellulose derivatives such as CMC but apparently have little or no effect on insoluble forms of cellulose (25, 26, 28). Although the mechanism of hydrolysis of cellulose by *M. verrucaria* is considered to be unienzymatic (31), evidence is accumulating that several enzyme systems are involved in the decomposition of native cellulose by other cellulolytic organisms. On the basis of extensive studies on microbial decomposition of cellulose, Reese and coworkers (24, 25, 26) have postulated the existence of a C_1 enzyme that acts on native cellulose to release long chains of anhydro-glucose units and a Cx enzyme that can split the β 1,4 anhydroglucose chains into smaller units to form cellobiose. Furthermore, the Cx enzyme system may itself consist of several different components. (24). Results of studies on the cellulase of rumen bacteria by Halliwell (9) also indicate that more than 1 enzyme may be involved in the hydrolysis of cellulose by these organisms. Thus, it would not be possible to conclude that *P. solanacearum* is a cellulolytic organism solely because the Cx enzyme is formed in culture and in diseased plants (11). However, examination of transverse sections of infected stems with a polarizing microscope showed that vessel walls in vascular bundles invaded by *P. solanacearum* were no longer birefringent. A similar loss in birefringence was noted in sections from cuttings placed in solutions of Myrothecium cellulase. This is considered to be strong evidence that the wilt bacterium produces the C_1 enzyme or a related enzyme capable of attacking native cellulose. These observations on the production of cellulolytic enzymes by *P. solanacearum* are of particular significance since there has been little evidence that bacterial plant pathogens produce cellulolytic enzymes except for the report by Ammann (1) of the formation of the Cx enzyme by 1 isolate of *Erwinia carotovora* (Jones) Holland.

Although it was concluded from these studies that pectic and cellulolytic enzymes do not play a significant role in wilting, it was possible to demonstrate that they are important in the decomposition of host tissues. The stem tissues of tomato cuttings were rapidly macerated when they were immersed in culture filtrates that contained PME, PG, and the Cx enzyme. This maceration was more rapid and extensive than that which occurred in solutions of Pectinol 100D or a purified PG. In addition to being formed in culture, PG and the Cx enzyme were also produced in infected plants (11). Confirmation that the enzymes produced in diseased plants were active in host tissues was obtained in histological studies of infected plants and cuttings placed in culture filtrates. From these observations it could be concluded that pectic and cellulosic constituents of the cell walls were decomposed by the enzyme systems of the pathogen. Thus, the PG and Cx enzymes of *P. solanacearum* partially fulfill the criteria for vivotoxins as defined by Dimond and Waggoner (6).

Differences were noted between the highly pathogenic F-1 and the weakly pathogenic B-2 strain with respect to levels of PME, PG, and the Cx enzyme formed in culture. However, additional studies will be necessary to determine whether levels of enzyme production can be positively correlated with the pathogenicity of a given strain.

The rapid circumferential and longitudinal spread of the bacteria in the host is undoubtedly facilitated by the disintegration of the tissues. To some extent, the extensive disorganization of vascular tissue may indirectly impede water movement and increase the severity of wilting that is mainly caused by vascular plugging with bacterial slime. In young and succulent plants with thin-walled cells and an abundance of pectic substances, the effects of pectolytic and cellulolytic enzymes are very pronounced, causing a rapid collapse of all stem tissues and hastening the death of infected plants. In older plants, with greater lignification of cell walls and proportionately smaller amounts of the pectic substances than in young plants, the destructive action of these enzymes is reduced in severity.

PLANT PATHOLOGY
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PHYTOPATHOLOGICAL NOTES

Toward A Uniform Seed Corn Cold Test.[1] M. V. Desai and C. S. Reddy. The Pythium cold test for seed corn is limited in its use because of non-uniform results in different seed laboratories and at different times in the same laboratory. This is due to uncontrolled variables, one of which is the pathogen that kills the seed under the cold test conditions. The soil contains numerous species of Pythium and strains of the same species; they act more or less the same in killing seed under adverse conditions of germination, but vary in virulence. Other factors being the same, the results vary with the abundance and the degree of virulence of the species and strains present. If only 1 virulent strain were present in a test, much greater uniformity could possibly be attained. Unpublished results of this hypothesis by the writers are promising, but such procedure would involve the use of the same inoculum by each seed laboratory (1 isolate), and in a form that is easily and cheaply maintained. This paper records one way to prepare such an inoculum.

Preliminary experiments on a controlled seed corn cold test showed that thoroughly washed and air-dried vermiculite was a satisfactory means of carrying the inoculum. Some nutrient must be added for growth, but this should be of such small amount that the stored inoculum is not a good medium for the growth of contaminants.

One liter each of potato, oat meal, pea, and lima bean extracts were prepared (from 200 g of potatoes and 100 g of each of the others). The potatoes and oatmeal were boiled, and the peas and lima beans autoclaved, for 1 hour at 15 lb. pressure. One g of agar was added to each liter of extract. Ten ml of potato extract were added to each of 16 tubes containing 5 g of washed air-dried vermiculite, and 10 ml of a 1:9 dilution of the same material were added to each of 16 additional tubes containing the same amount of vermiculite. The same procedure was carried out for each of the other extracts. The tubes were plugged, sterilized, cooled, and inoculated with a

[1] Journal Paper No. J-3307 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project No. 858.

virulent culture of Pythium isolated from seed killed during germination. Four tubes of each extract were incubated at 10°, 15°, and 20°C. The poorest growth was with pea extract. The best growth was made at 15° and 20°C and on vermiculite to which oatmeal extract had been added. On the basis of these results a similar experiment was conducted using vermiculite with oatmeal extract at 0.5, 1.0, and 2.0 per cent strength but omitting the agar. The most satisfactory growth was made on the vermiculite treated with 1 per cent oatmeal extract. Five g of vermiculite will absorb 10 cc of liquid.

Cultures of this Pythium species grown on 1 per cent oat extract held by vermiculite were lyophilized in an unsuccessful attempt to preserve the fungus. Staffeldt and Sharp[2] also found that Pythium sp. producing only sporangia and mycelia did not survive lyophilization.

Cultures of this same Pythium sp. were used for determining longevity at different moisture contents when dried at different temperatures. After inoculation the cultures were incubated 6 days at room temperature and divided for drying at 35–37°C, 29–31°C, and 24–26°C. The weight for each tube containing 5 g of air-dried (about 2 per cent moisture) vermiculite was recorded before adding 10 cc of extract so that moisture percentage could be determined at any time by weighing. When the weight was 14–16 per cent higher than the 5 g of air-dried vermiculite, the contents of the tube were poured into a sterile Petri plate and covered with filter paper. Sterile water was added until both the vermiculite and the filter paper were saturated. The viability of the culture and its pathogenicity were tested by allowing 48 hours for the culture to become active and then sowing the plate with alfalfa seeds. A viable and pathogenic culture will cause seed rot or seedling necrosis in 48–96 hours. This was repeated with cultures at 10–12 per cent and 6–8 per cent water.

Immediately after viability and pathogenicity were determined, 5 tubes from each category of moisture and drying temperature were sealed with paraffin wax paper and stored at 0°C. Tests were made 2 weeks and 6 weeks after storing, and subsequently at 2-month intervals.

The cultures did not survive drying at 35–37°C, but the experiment showed that, when dried at 29–31°C or 24–26°C and at moistures of 6–16 per cent, the cultures did not lose viability or pathogenicity, at least up to 8–10 months. With this development of a low-moisture vermiculite inoculum that has adequate longevity, the perfection of a cold test, uniform at any time or place, seems closer. The culture used in these experiments was obtained by testing the seed-rotting virulence of more than 200 isolates of Pythium sp. from killed kernels of seed corn that had been placed in soil under conditions for germination. A high percentage of the isolations could not be made to sporulate enough for species identification. It is hoped that this very virulent non-sporulating isolation will remain stable over a long period, and that the concentration of the inoculum, when fitted with a revised method of cold testing (now under experimentation), will ensure uniform results from cold tests. Department of Botany and Plant Pathology, Iowa State College, Ames, Iowa.

[2] Staffeldt, E. E., and E. L. Sharp. 1954. Modified lyophil method for preservation of Pythium species. Phytopathology 44: 213-215.

A Method for Inoculating Single Wheat Leaves With More Than One Race of Puccinia graminis f. sp. tritici.[1] J. R. Geis, M. C. Futrell, and W. N. Garrett.[2] A method of inoculating a single wheat leaf with one to several races of *Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E. Henn. is being used successfully at Beltsville, Maryland, and at College Station, Texas. Of the several methods suggested by Dr. E. B. Lambert, "patch inoculation" as described in this paper has given the best results. The materials used are ⅛-in. squares of filter paper and 3-in. × ½-in. pieces of Cellophane tape. To facilitate handling the patch, both ends of the tape are folded back from a crease made in the center. With these materials a patch inoculation is made as follows: Immerse the filter paper in a water suspension of spores, remove, affix to Cellophane tape near the center crease, and position over the spot on the leaf where infection is desired. Press the tape lightly but firmly together around both sides of the leaf so that the filter paper makes full contact with the leaf surface (Fig. 1,A). It apparently makes no difference whether the filter paper is on the upper or lower surface of the leaf. The area of the leaf to be inoculated should first be marked with India ink or other waterproof material.

Plants with patches applied should be kept in a humidity chamber overnight unless a larger piece of moistened filter paper has been placed between the tape and the infection pad. Under field conditions, patches should be applied to plants late on a humid afternoon, using wider Cellophane tape. The morning after inoculation the patches should be removed, first allowing any moist tape to dry out for easier removal.

More than 100 patches have been tried under greenhouse conditions; over 95 per cent resulted in infection. Fig. 1.B shows leaves of each of 2 lines of wheat inoculated in the greenhouse by the above method with 3 races of *P. graminis* f. sp. *tritici*. The technique has been found particularly useful for inoculating F_1 plants and critical F_2 populations where the reaction is wanted of each plant to more than one race.

A number of inoculation methods have been used at College Station during the past five years in studies on the overwintering and spread of stem rust under field conditions. Hypodermic needles, glass-jar incubators, and several other devices result in good infec-

[1] Cooperative investigations of the Crops Research Division, ARS, USDA, and the Texas Agricultural Experiment Station.

[2] Agronomist and Pathologist, Crops Research Division, ARS, USDA; and Graduate Student, Agricultural and Mechanical College of Texas, respectively.

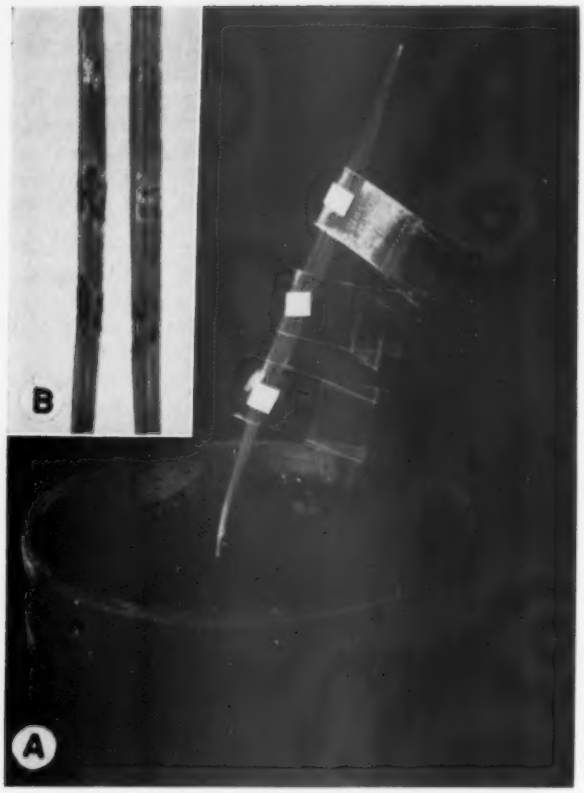

FIG. 1. A) Patch inoculation of a seedling plant with three races of *Puccinia graminis* f. sp. *tritici*. B) Reactions of a leaf of each of 2 lines of wheat inoculated by the above method with 3 races (29, 139, and 56).

tion but produce a smear of many pustules on the stem or leaf. In nature, however, isolated pustules usually start overwintering centers. The patch inoculation method was tried in the field during the fall of 1956. The resulting infection more closely simulated a single pustule than infections resulting from other methods.

The patch and glass-jar methods were compared under field conditions at College Station. One-inch Cellophane tape was used for the patch inoculations. A total of 72 patch and 96 glass-jar inoculations resulted in 90 and 100 per cent infection, respectively. The tapes had no toxic effects on the rust or host plant.

Inoculations by either method should be made on a humid afternoon when good soil moisture is present and night temperatures are above 60°F, or both methods will fail under field conditions. During periods of rain or extremely high humidity in fields in south Texas, the adhesive sometimes separated from the Cellophane.

It is believed that the patch inoculation technique can be modified for uses on other plant parts and that several other organisms may be handled in a similar way.—Agricultural Research Service, U. S. Department of Agriculture, Department of Plant Physiology and Pathology, Texas Agricultural Experiment Station, College Station.

Additional evidence for seed-borne mycelium of Sclerotinia sclerotiorum associated with clover seed. Charles M. Leach. Sclerotia of *Sclerotinia sclerotiorum* (Lib.) d By. sensu Purdy[1] mixed with clover seed are important in dissemination of this pathogen. Eriksson[2] and Pape[3] suggested that the clover Sclerotinia is also spread with seed as ascospores and mycelia. The only supporting evidence is a report by Justham and Ogilvie,[4] who isolated the fungus from a single surface-sterilized clover seed. Noble[5] is of the opinion that there is little evidence that this Sclerotinia is transmitted with seed as mycelia; she considers that it occurs only as sclerotia mixed with clover seed.

In a study of the fungi associated with Oregon-grown clover seed[6] (*Trifolium pratense* L., *T. hybridum* L., *T. repens* f. *giganteum* Lagr.-Foss, and *T. incarnatum* L.), surface-disinfected and untreated seed were placed on 2 per cent malt extract agar. Surface disinfection[7] was with a freshly prepared mixture of 5.25 per cent sodium hypochlorite, 95 per cent ethanol, and water (4:1:8). Each sample tested consisted of 200 untreated and 200 surface-disinfected seeds.

S. sclerotiorum was observed growing from seed (Fig. 1) of 8 of the 19 samples of *T. incarnatum*. Infestation of seed from untreated samples was 0.5–10.0 per cent. After surface disinfection the fungus was detected in only 3 samples. When untreated seed from infested samples were stored 2 years in corked vials at room temperature, no Sclerotinia was detected.

The fungus isolated from crimson clover seed was identified as *Sclerotinia sclerotiorum* (Lib.) d By. sensu Purdy on the basis of the morphology of mycelia, sclerotia, and apothecia.

Since none of the colonies of *S. sclerotiorum* growing from seed plated on agar originated from sclerotia, they must have arisen from either ascospores or mycelia associated with the seed. Furthermore, since surface disinfection reduced but did not completely eliminate viable Sclerotinia from infested seed, it is probable that the fungus had penetrated into the seed as hyphae. There is the possibility that the fungus grew

[1] Purdy, L. H. 1955. A broader concept of the species Sclerotinia based on variability. Phytopathology 45: 421-427.

[2] Eriksson, J. 1880. Um klofverroten med sarskiltafseende pa dess upptradanda ivart fadernesland aren 1878-1879. Kongliga Landtbruksakademien, Stockholm. Handlingar och Tidskrift No. 1, p. 16 (Abstract in Botan. Centr. 1: 296-297, 1880).

[3] Pape, H. 1931. Das Auswintern des Klees. Mitt. deut. Landwirtsch. Ges. 46(12): 233-235, (13): 257-259. (Abstracted in Rev. Appl. Mycol. 10: 669-670. 1931.)

[4] Justham, M., and L. Ogilvie. 1950. Clover rot investigations. Ann. Appl. Biol. 37(2): 328.

[5] Noble, Mary. 1948. Seed-borne diseases of clover. Trans. British Mycol. Soc. 30: 85-91.

[6] Leach, C. M. 1956. Fungi associated with Oregon-grown clover seed. (Abst.) Phytopathology 46: 637.

[7] Leach, C. M. 1955. Equipment and methods for the isolation of pathogens from clover seed. Phytopathology 45: 94-96.

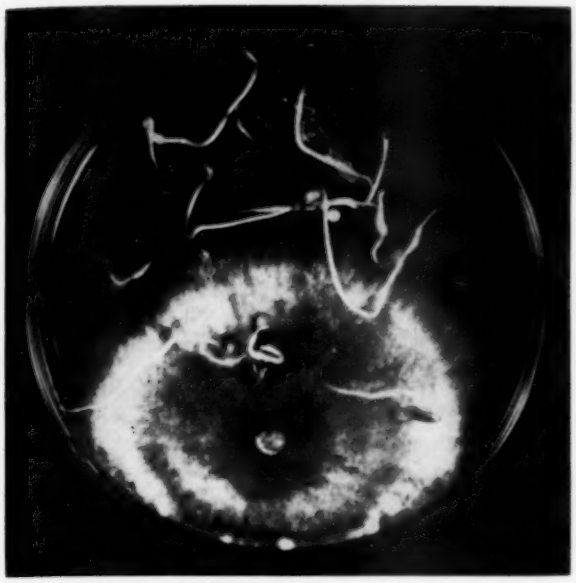

FIG. 1. Five-day-old colony of *Sclerotinia sclerotiorum* (Lib.) d By. sensu Purdy, growing from a crimson clover seed (Sclerotinia is the larger colony).

from ascospores associated with the untreated crimson clover seed, but this is highly unlikely, for apothecia are rarely found during the blossoming period of crimson clover. Of the 4 clover species examined, crimson clover seed alone was infested, as might be expected under Oregon conditions.

The occurrence of seed-borne mycelia of *S. sclerotiorum* with Oregon-produced seed of *T. incarnatum* further supports the contention of earlier plant pathologists that this fungus may be transmitted as mycelia with clover seed. This form of seed transmission should be considered in any program for the phytosanitary certification of crimson clover seed.

Loss of viability of the seed-borne mycelium in a 2-year storage period offers a possible means of obtaining seed free of this pathogen if chemical treatments are not effective.—Department of Botany and Plant Pathology, Agricultural Experiment Station, Oregon State College, Corvallis, Oregon.

A New and Virulent Culture of Oat Stem Rust.[1] Donald M. Stewart and Ralph U. Cotter.[2] An isolate of *Puccinia graminis* Pers. var. *avenae* (Eriks. & Henn.) Seym. that causes heavy infection at moderate temperature on varieties and lines of oats with the 3 major known types of stem-rust resistance was obtained from Rodney oats, collected by Dr. Neal F. Jensen near Ithaca, New York, on August 2, 1957, and sent by Dr. George C. Kent to the Cooperative Rust Laboratory at St. Paul, Minnesota, for identification. Because this isolate resembles race 13 on Richland, Minrus, and Jostrain, long used as standard differentials, it is provisionally designated 13A. However, the new isolate differs sharply from race 13 on 4 additional varieties of oats, as shown in Table 1.

It is evident from Table 1 that race 13A causes normal infection at 70–75°F on varieties with the Richland (gene A), White Russian (gene D), and Hajira, or "Canadian" (linked genes BC), types of resistance, and on varieties with any combination of the 3. It is the only isolate identified at the Cooperative Rust Laboratory that has this ability. It is generally more virulent than any of the 2102 uredial isolates identified from 1682 collections made in 1954–1957. All of the varieties listed in Table 1, and several other new varieties and lines, were inoculated during that period, in the same manner as the standard differential varieties, with all collections of oat stem rust from which race identifications were made. It appears, therefore, that 13A is of recent origin.

Race 13A is generally more pathogenic than any other known race on varieties with the 3 major types of resistance utilized in breeding for resistance in United States. Of the prevalent races, race 8 attacks Richland but not White Russian or New Garry; race 7 attacks White Russian but not the other 2; races 6 and 13 attack both Richland and White Russian but cause susceptible reactions on New Garry only at temperatures of 80–85°F. In 1954 Welsh and Johnson[3] found that an isolate designated as 7A also could attack varieties with the "Canadian" (Rodney) type of resistance at moderate as well as at high temperature, but 7A does not attack Richland or New Garry. Therefore, isolate 13A alone has a pathogenic ability equivalent to races 7, 7A, 8, 6, and 13 combined.

Race 13A was first found in a barberry-infested area, as was true of the first collections of races 8,[4] 7,[5] and 6 in the United States. The first 2 finally became independent of barberry, widespread, prevalent, and destructive; but races 6 and 13 have not yet become established outside of barberry areas.

Whatever the future of 13A may be, it is evident that it appears to have a combination of the genes for virulence of several races and is potentially dangerous. It is therefore being studied thoroughly in the greenhouse, during the winter season only, with proper pre-

[1] Cooperative investigations between the U. S. Department of Agriculture and the Minnesota Agricultural Experiment Station. Paper No. 3874, Scientific Journal Series, Minnesota Agricultural Experiment Station.

[2] Plant Pathologists, Plant Pest Control Division, Agricultural Research Service, U. S. Department of Agriculture.

[3] Welsh, J. N., and T. Johnson. 1954. Inheritance of reaction to race 7A and other races of oat stem rust, Puccinia graminis avenae. Can. J. Botany 32: 347-357.

[4] Stakman, E. C., and W. Q. Loegering. 1944. The potential importance of race 8 of Puccinia graminis avenae in the United States. Phytopathology 34: 421-425.

[5] Stakman, E. C., and W. Q. Loegering. 1951. Physiologic races of Puccinia graminis in the United States in 1950. U. S. Dept. Agr., Agr. Res. Admin., Bur. Entomol. Plant Quarantine, Bur. Plant Ind. Soils and Agr. Eng., and Minnesota Agr. Expt. Sta. Mimeograph, 16 p.

TABLE 1.—*Seedling reaction to race 13 and to 13A at 70–75°F*

Variety or selection	Reaction[a] to races 13	13A	Resistant genotype
Standard differential varieties			
Richland, C. I. 787	S	S	A
Minrus, C. I. 2144	S	S	D
Jostrain, C. I. 2660	M	M	
Supplemental trial varieties			
Rodney, C. I. 6661	R	S	BC
Burnett, C. I. 6537	R	S	BCD
New Garry, C. I. 6662	R	S	ABC
Minn. 53 Ag. 313, C.I.7145	R	S	ABCD

[a] S = susceptible; M = mesothetic; R = resistant

cautions to prevent its escape.—Plant Pest Control Division, Agricultural Research Service, U. S. Department of Agriculture, and the Minnesota Agricultural Experiment Station, St. Paul, Minnesota.

An Efficient Method of Obtaining Single Conidial Isolates of Powdery Mildew. W. C. Schnathorst. To separate pure strains of *Erysiphe cichoracearum* DC. ex Mérat from mixtures occurring naturally or artificially on the same plant, or to test for homo- and heterothallism in this fungus, it is necessary to isolate either single ascosporic or single conidial lines of the organism. Single ascosporic and conidial lines of powdery mildews have been obtained and studied by several workers: Cherewick,[1] Powers and Moseman,[2] Schnathorst,[3] and Yarwood.[4] However the writer has found no description of an efficient method of obtaining single conidial cultures of powdery mildews. The writer failed to establish single conidial lines when conidia on small blocks of water agar in Petri dishes were individually transferred to detached leaves floated on distilled water. No colonies were obtained when conidia adhering to the tips of glass needles were individually wiped on the surface of detached leaves. This paper describes a technique whereby the theoretical maximum of successful single conidial inoculations is approached.

Detached lettuce (*Lactuca sativa* L.) or wild lettuce (*L. serriola* L.) leaves were lightly sprayed with distilled water by means of a hand atomizer. Conidia of *E. cichoracearum* that had been dusted on water agar in a Petri dish were singly transferred to the point of a glass needle by the surface tension of a film of water on the tip of the needle. The tip of the needle was then carefully inserted into a distilled-water droplet (1 mm maximum diameter) on a detached leaf. The leaves were floated on distilled water in Petri dishes and incubated at 22°C with illumination of 300 ft-c at the leaf surface. Excessive light reflection from the needle and droplet, besides the fact that the spore is hyaline, prevented observing the actual transfer of the spore under a binocular dissecting microscope. Theoretically, the spore should be snapped by surface tension from the tip of the needle onto the water droplet. When conidia of the 2 strains of mildew studied (lettuce and wild lettuce strains) were floated only on distilled water, percentage of germination was usually 50 per cent or less.[3] This indicated that germination was not impaired when spores were placed on water droplets on leaf surfaces. It was thought that with evaporation of the droplets the spores would probably lower to the surface of the leaf in a few hours with subsequent infection. It was found that at most only 50 per cent of the spores that germinated actually formed a mildew colony.[3] The theoretical maximum of success for single conidial inoculations under these conditions was therefore 0.5×50, or 25 per cent. From 175 single conidial inoculations of detached leaves of *L. sativa* and *L. serriola* with both strains of mildew by the method described, 26 colonies were obtained, which is about 60 per cent of the number theoretically possible. In one series of 50 single-spore transfers, 88 per cent of the number of successful inoculations theoretically possible was obtained.

This method may be applicable to powdery mildew fungi that are quite sensitive to free moisture. Conidia on the surface of distilled water showed a substantially higher percentage of germination than did conidia on leaves or glass, where condensation inundated them, apparently greatly interfering with germination.[3] Department of Plant Pathology, University of California, Davis.

[1] Cherewick, W. J. 1944. Studies on the biology of Erysiphe graminis DC. Can. J. Research 22: 52-86.

[2] Powers, H. R., Jr., and J. G. Moseman. 1957. Pathogenic variability within cleistothecia of Erysiphe graminis. Phytopathology 47: 136-138.

[3] Schnathorst, W. C. 1957. Etiology of lettuce powdery mildew (Erysiphe cichoracearum DC. ex Mérat). Ph.D. Thesis, Univ. of Calif.

[4] Yarwood, C. E. 1935. Heterothallism of sunflower powdery mildew. Science 82: 417-418.

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(1712-1783)

NEWS

Editor: K. W. KREITLOW, Plant Industry Station, Beltsville, Md.

Director James G. Horsfall, Connecticut Agricultural Experiment Station, received an honorary Doctor of Science degree from the University of Vermont, Burlington, Vermont, on June 15, 1958. The degree was awarded for his outstanding contributions to agriculture, industry and science in the field of plant pathology. This award is also being granted during the 50th anniversary year of the founding of the American Phytopathological Society. The citation was read and presented by Dr. Thomas Sproston, Plant Pathologist of the University of Vermont.

Congratulations to plant pathologist Thorvaldur Johnson, Chief, Canada Department of Agriculture Research Laboratory, Winnipeg, Manitoba. Dr. Johnson was presented with the Elvin Charles Stakman Award for outstanding research in cereal diseases at the Cap and Gown Day Convocation of the University of Minnesota, May 22, 1958.

A grant has been made to the University of Maryland by the Rockefeller Foundation in support of a study on the biology and control of plant parasitic nematodes.

Forest Pathologist Dow V. Baxter on sabbatical leave from the Pathology Laboratory in Forest Practice, School of Natural Resources, University of Michigan, will return to Alaska for his 19th field season to be spent in the Territory. He expects to be at the Department of Plant Pathology at the University of California in Berkeley and at Riverside during most of the fall term.

A Symposium on Plant Tumors was held before the National Academy of Sciences in New York, November, 1957. The papers given have been published in the April, 1958 number of the Proceedings of the National Academy of Sciences. This is possibly the first time a plant disease has been the subject of a National Academy Symposium and in a way, commemorated the 50th Anniversary of the published description of *Bacterium tumefaciens*, the cause of crown-gall. Participants and their topics were as follows: A. J. Riker, Introduction; Philip R. White, A tree tumor of unknown origin; Armin C. Braun, A physiological basis for autonomous growth of the crown-gall tumor-cell; Richard M. Klein, Activation of metabolic systems during tumor-cell formation; A. C. Hildebrandt, Stimulation or inhibition of virus infected and insect-gall tissues and single-cell clones; L. M. Black, Wound-tumor; A. J. Riker, Conclusions.

Phytopathologist Paul C. Duffield was appointed Assistant Professor in the Department of Botany and Plant Pathology, Kansas State College, June 1, 1958. Dr. Duffield will work on forage crop diseases in Kansas. He was formerly the Research Coordinator in the Rockefeller Mexican Agricultural Program.

The 1st International Meeting on Seed Pathology was held in Cambridge, May 12-17, 1958, under auspices of the International Seed Testing Association. Main object of the meeting was to discuss means of standardizing testing techniques for seed-borne pathogens. Other subjects included effect of seed dressings on tests and interpretation of test results in terms of field performance.

Mycologist Edith Cash retired May 29 after more than 45 years service in the mycological work of the U.S. Department of Agriculture.

Chemical factors responsible for disease resistance in plants will be studied by Robert N. Goodman who plans to spend one year at the Institute for Special Botany, Swiss Federal Institute of Technology, Zurich, Switzerland. Dr. Goodman, Associate Professor of Horticulture, University of Missouri, is the recipient of fellowships awarded by the Simon Guggenheim Foundation and the Lalor Foundation. His research will be under the direction of Dr. Ernst Gäumann.

Nematologist Benjamin G. Chitwood, formerly Chief Nematologist of the Florida State Plant Board has accepted a position with the Kaiser Foundation, Berkeley, California.

Phytopathologist G. M. Watkins, Head, Department of Plant Physiology and Pathology, Texas A and M College, since 1950, will terminate his present assignment to become Dean of the College of Agriculture effective July 1.

Plant Pathologist William A. Feder, Agricultural Research Service, U. S. Department of Agriculture, Orlando, Florida, will be on leave at the Polytechnic Institute, London, England, beginning September 10, 1958. He has received a Senior Postdoctoral Fellowship to enable him to collaborate with Dr. C. L. Duddington in a study of certain nematode-inhabiting fungi.

Phytopathologist L. M. Massey, internationally-known plant pathologist, will retire officially from the Cornell University faculty June 30. Unofficially, the professor and former department head will continue his research on fungicides and rose diseases. Dr. Massey's work at Cornell spans almost a half century. He came to Cornell for graduate work in 1912. He left for a short time (1918-19) on leave of absence for war relief work as a pathologist with the United States Department of Agriculture. From 1922 till 1950, he was head of Cornell's plant pathology department. He resigned from the position to devote full time to research and teaching advanced courses. During his early years, 1915-25, Dr. Massey did intensive research on gladiolus diseases. He was the first to recognize many of the diseases of that flower. Since then, he has worked with roses, both as a vocation and avocation. Many of the disease-control measures he developed are still common practices. He also acted as consulting pathologist for the American Rose Society and director of their disease control campaign. In 1947, the Society awarded him its highest honor, a gold medal in recognition of his contributions. Dr. Massey is one of the few authorities on the effect of industrial fumes on vegetation, and, in that capacity, has served as a consultant in many areas of the United States.

Plant Pathologist H. N. Racicot of the Ottawa, Canada laboratory retired from the Division of Botany and Plant Pathology on March 9.

Deaths—Dr. Raymond F. Poole, President of Clemson College, Clemson, South Carolina, died June 6 at the age of 65. Dr. Poole was assistant plant pathologist at Clemson College from 1915 to 1917. He also served for a brief period as plant pathologist at the New Jersey Agricultural Experiment Station. From 1926-1940, he was on the staff of the North Carolina State College. In 1940, Dr. Poole was made president of Clemson College and he was serving in this capacity when he died. Despite his administrative duties, Dr. Poole maintained an interest in plant pathology until his death.